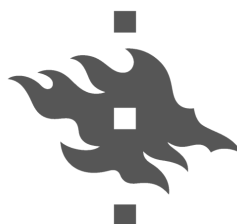




Minerva Foundation Institute  
for Medical Research



University of Helsinki

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# NOVEL MOLECULAR REGULATORS OF ADIPOSE TISSUE METABOLISM

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Raghavendra Mysore

Minerva Foundation Institute for Medical Research  
and  
Faculty of Medicine, Department of Internal Medicine and Doctoral  
program in Biomedicine, University of Helsinki, Finland

ACADEMIC DISSERTATION

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**Supervisors:**

Professor Vesa Olkkonen, Ph.D

Minerva Foundation Institute for Medical Research, Helsinki, Finland

University of Helsinki, Helsinki, Finland

And

Nidhina Haridas P A, Ph.D

Minerva foundation institute for medical research

Biomedicum, Helsinki, Finland

**Reviewers**

Jacob Hansen, Ph.D

Associate professor, Department of Biology

University of Copenhagen, Denmark

And

Tarja Kokkola, PhD

Docent, University of Eastern Finland

**Opponent**

Professor Jussi Pihlajamäki, MD, PhD

Professor of Clinical Nutrition.

Clinical Nutrition and Obesity Center,

Kuopio University Hospital, Kuopio, Finland

**Custos**

Professor Hannele Yki-Jarvinen (MD, FRCP)

Professor of medicine

Department of medicine, University of Helsinki, Finland

**Cover image:**

Comprehensive representation of thesis study illustrating several screenings (miRNA and gene expression) from obese and lean human adipose tissue and cultured adipocytes.

Lipid droplet staining of 3T3-L1 adipocytes with Bodipy (green) and nuclei with DAPI (blue).

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To my teachers

Thank you for your trust and inspiration

ಗುರುಬ್ರಹ್ಮಾ ಗುರುವಿಷ್ಣುಃ ಗುರುದೇವೋ ಮಹೇಶ್ವರಃ

ಗುರುಃ ಸಾಕ್ಷಾತ್ ಪರಬ್ರಹ್ಮಾ ತಸ್ಮೈ ಶ್ರೀಗುರವೇ ನಮಃ

Teacher is an absolute representation of Brahma (god of creation),  
Vishnu (god of Sustenance) and Shiva (god of annihilation).  
He creates, sustains knowledge and destroys the weeds of ignorance.  
My Salutation to such a Guru, who is verily the Supreme God.

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## ORIGINAL PUBLICATIONS

This thesis is based on the following original research articles, which are referred to in the text by their Roman numerals. The original publications have been reproduced with the permission of the copyright holders.

- I. **Mysore R**, Zhou Y, Sädevirta S, Savolainen-Peltonen H, Nidhina Haridas PA, Soronen J, Leivonen M, Sarin AP, Fischer-Posovszky P, Wabitsch M, Yki-Järvinen H, Olkkonen VM. MicroRNA-192\* impairs adipocyte triglyceride storage. *Biochim Biophys Acta*. 2016 Apr;1861(4):342-51. doi: 10.1016/j.bbali.2015.12.019. Epub 2015 Dec 30. PMID: 26747651.
- II. **Mysore R**, Ortega FJ, Latorre J, Ahonen M, Savolainen-Peltonen H, Fischer-Posovszky P, Wabitsch M, Olkkonen VM, Fernández-Real JM, Nidhina Haridas PA. MicroRNA-221-3p regulates Angiopoietin-like 8 (ANGPTL8) expression in adipocytes. *J Clin Endocrinol Metab*. 2017 Nov 1;102(11):4001-4012. doi: 10.1210/jc.2017-00453. PMID: 28938482
- III. **Mysore R**, Liebisch G, Zhou Y, Olkkonen VM, Nidhina Haridas PA. Angiopoietin-like 8 (Angptl8) controls adipocyte lipolysis and phospholipid composition. *Chem Phys Lipids*. 2017 Oct;207(Pt B):246-252. doi: 10.1016/j.chemphyslip.2017.05.002. Epub 2017 May 18. PMID: 28528274

## **ABBREVIATIONS**

36B4	Acidic ribosomal phosphoprotein
ACC	Acetyl-CoA carboxylase
Acetyl-CoA	Acetyl Coenzyme A
ACSL	Acyl-coenzyme A synthetase long chain
ADIPOR	Adiponectin receptor
AGO	Argonaut
ALDH3A2	Aldehyde dehydrogenase 3 family A2
ALP	Alkaline phosphatase
ALT	Alanine transaminase
	Adenosine monophosphate activated protein kinase
AMPK	
ANGPTL8	Angiopoetin like protein 8
AP2	Adipocyte protein 2
APOH	Apolipoprotein H
AST	Aspartate transaminase
AT	Adipose tissue
ATGL	Adipocyte triglyceride lipase
BAT	Brown adipose tissue
BMI	Body mass index
C9	Complement component 9
cAMP	Cyclic adenosine monophosphate
CAV	Caveolin
CCL2	C-C Motif Chemokine Ligand 2
Cpt1a	Carnitine Palmitoyltransferase 1A
Cpt1b	Carnitine Palmitoyltransferase 1B
CVD	Cardio vascular disease
CYP3A5	Cytochrome P450 3A5
DAG	Diacylglycerol acyltransferase
DGAT1	Diacyl glycerol O-Acyltransferase-1
DGCR8	DiGeorge syndrome critical region 8
DNL	De novo lipogenesis



ERK5	Extracellular-Signal-Regulated Kinase 5
FA	Fatty acid
FABP	Fatty acyl binding protein
FAS	Fatty acid synthase
FFA	Free fatty acid
FXR	Farnesoid X receptor
GLUT	Glucose transporter
GPAT1	Glycerol-3-phosphate acyltransferase-1
GTP	Guanosine triphosphate
HDL	High density lipoprotein
HIF1 $\alpha$	Hypoxia-inducible factor 1 $\alpha$
HSL	Hormone sensitive lipase
IL1 $\beta$	Interleukin 1 beta
IL1R2	Interleukin 1 receptor type 2
IR	Insulin resistance
JNK	c-Jun N-terminal kinases
KD	Knock down
KLF6	Krüppel-like factor 6
LAMP2	Lysosomal associated membrane protein 2
LD	Lipid droplet
LPL	Lipoprotein lipase
MAG	Monoacyl-glycerol
MCM	Macrophage conditioned media
MCP1	Monocyte chemoattractant protein 1
miRNA	MicroRNA
MOGAT1	Monoacylglycerol O-Acyltransferase-1
mRNA	Messenger RNA
MSR1	Macrophage scavenger receptor 1/ scavenger receptor A
MUFA	Monounsaturated fatty acid
NEFA	Non-esterified fatty acid
NF-kB	Nuclear factor-kB
nt	Nucleotide
oxLDL	Oxidized low density lipoprotein

PABP	Poly(A)-binding protein
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
Pgc1a	Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha
PGE2	Prostaglandin E2
PKA	Protein kinase A
PLA2G7	Phospholipase A2 G7
PLIN	Perilipin
PNPLA3	Patatin-like phospholipase domain containing 3
PPAR	Peroxisome proliferator-activated receptor
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SAT	Subcutaneous adipose tissue
SCARB1	Scavenger receptor class B, type I
SCD	Stearoyl coenzyme A desaturase gene
SDHA	Succinate dehydrogenase complex, subunit A
SGBS	Simpson Golabi Behmel syndrome
SREBF1	Sterol regulatory element binding protein-1 gene
SREBP	Sterol regulatory element-binding protein
SVF	Stromal vascular fraction
sWAT	Subcutaneous white adipose tissue
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TG	Triglycerides
TNF- $\alpha$	Tumor necrosis factor-alpha
TRBP	TAR RNA binding protein
UCP1	Uncoupling protein 1
UTR	Untranslated region
VAT	Visceral adipose tissue
VLDL	Very low-density lipoprotein
WAT	White adipose tissue

## **ABSTRACT**

Adipose tissue is distributed across the body as a characteristic depot to serve timely energy demands. Adipocytes are the functional units of adipose tissue (AT) maintain glucose and lipid homeostasis, a robust physiological system regulated precisely by complex network of molecular stimuli to achieve the energy demands in both fed and fast state. Adipocytes are most versatile cells which efficiently synthesise fat, store fatty acids safely and release them upon requirement through three well-coordinated processes termed lipogenesis, lipid storage and lipolysis. These processes are highly sensitive to nutritional and hormonal stimuli and respond via proteins and/or miRNA regulation. Dysregulation leads to various metabolic complications like obesity, insulin resistance (IR), type 2 diabetes (T2D), cardiovascular disorders (CVD), metabolic syndrome and cancer. AT related complications are physiologically interlinked and can affect other metabolic organs like liver, heart, muscle etc. Hence, it is important to understand the mechanisms regulating adipocyte differentiation, lipid storage, lipolysis and signalling to improve our understanding on adipose tissue in metabolism. This thesis describes the newly identified miR-192\* as regulator of adipocyte lipid metabolism, miR-221-3p as a novel regulator of ANGPTL8 in human adipose tissue and investigates the function of ANGPTL8 in cultured adipocytes.

We studied the expression of miR-192\* in visceral adipose tissue (VAT) of two obese subject groups. In VAT of cohort, I (morbidly obese group undergoing bariatric surgery) we found negative correlation between miR192\* with serum triglyceride (TG) and positive correlation with high-density lipoprotein (HDL) concentration. In cohort II (less obese patients) miR-192\* negatively correlated with the body mass index (BMI). Overexpression of miR192\* in cultured adipocytes revealed reduced expression of the main adipocyte differentiation marker proteins perilipin 1 and aP2 (adipocyte protein 2) with marked reduction in TG content. Transcriptome profiling of adipocytes expressing miR-192\* revealed impact on central genes of lipogenic pathway. Altered mRNA expression

of these genes were validated by qPCR and western blotting. We showed that SCD (stearoyl coenzyme A desaturase-1) and ALDH3A2 (aldehyde dehydrogenase 3 family A2) are direct targets of miR-192\*.

In study II, we addressed the regulation of ANGPTL8 by miR-221-3p under inflammatory stimuli. An interesting positive correlation was observed between mRNA expressions of ANGPTL8 and ADIPOQ (adiponectin), GLUT4 and fatty acyl synthase diacylglycerol O-acyltransferase 1 in subcutaneous (SAT) and visceral AT (VAT). ANGPTL8 mRNA expression was significantly reduced upon inflammation-mimicking conditions with concomitant induction of miR-221-3p expression in cultured adipocytes. We showed that miR-221-3p physically targets the 3'UTR of ANGPTL8 and reduces its protein expression. VAT biopsy analysis from obese subjects (cohort II, n=19) and SAT biopsies from subjects varying from lean to obese (cohort III, n=69) showed a negative correlation trend between ANGPTL8 and miR-221-3p. Significant negative correlation was found between ANGPTL8 and miR-221-3p expression in morbidly obese subgroup of SAT samples (cohort III, n=22) before bariatric surgery, which interestingly disappeared after 2-year post surgery weight loss resulting in a significant reduction of miR-221-3p. ANGPTL8 negatively correlated with the AT inflammatory marker phospholipase A2 G7, while miR-221-3p showed a significant positive correlation with this inflammatory marker.

In the last part, we studied the intracellular function of ANGPTL8 using lentiviral shRNA based stable knockdown method in cultured adipocytes. This resulted in a moderate but significant reduction of TG accumulation. The lipidome analysis presented a decrease in alkylphosphatidylcholines (alkyl-PCs) and phosphatidylethanolamine (PE) plasmalogens, as well as saturated PCs and PEs. Adipocytes devoid of ANGPTL8 revealed enhanced lipolysis, and genes encoding ANGPTL4 and leptin, inducers of lipolysis, as well as Cpt1a (Carnitine Palmitoyltransferase 1A), Cpt1b (Carnitine Palmitoyltransferase 1B), and PGC-1 $\alpha$  involved in FA oxidation, were upregulated. Pharmacological treatments inducing lipolysis also reduced the expression of ANGPTL8 mRNA.

These studies together describe the role of miR-192\* in AT lipid metabolism, relation between miR221-2p and inflammation in regulating ANGPTL8 and a functional role of ANGPTL8 in cultured adipocytes.

## 1. INTRODUCTION

Over the past century, obesity has emerged as a major public health concern affecting one third of adults and one in five children across the globe. Obesity is a major risk factor for the progression of IR, T2D and strongly associated with CVD, NAFLD (non-alcoholic fatty liver disease), dyslipidaemia and several cardiometabolic complications<sup>1</sup>. Positive energy balance is the major cause for obesity generally driven by intake of high-calorie diet, sedentary life combined with genetic predisposition and other socioeconomic factors. AT buffers the energy fluctuation by safely storing fat during abundance and releasing it during starvation. It also performs endocrine and various physiological functions. AT is a multifarious tissue composed of preadipocytes and mature adipocytes along with adipocyte precursor cells, numerous microvascular and immune cells. Anatomical distribution of AT plays a vital role in determining its impact on metabolism. AT can be classified into central AT (visceral and subcutaneous fat mass from upper abdominal region) and peripheral AT (femoral fat from hip and gluteal region). Excess central fat, especially VAT, contributes to a higher rate of lipolysis, IR and proinflammatory cytokines relative to SAT<sup>2</sup>. It has been reported that visceral fat cells *in vitro* show elevated lipid synthesis and lipolysis compared to subcutaneous adipocytes<sup>2-4</sup>. VAT secretome contributes to elevated serum TG concentrations which may result in hepatic insulin resistance and/or steatosis<sup>5,6</sup>. miRNAs are small non-coding RNA molecules with the length of 19-23 nucleotides. They regulate gene expression by targeting mRNA. Resulting in degradation or translational repression<sup>7</sup>. miRNAs have been reported to play a significant role in adipose tissue biology. Several miRNAs, including miR-143, miR-30c, miR-148a, miR-26a and b promote adipogenesis<sup>8-11</sup> and miR-27b, miR-130 and miR-375 have been reported to inhibit adipogenesis by targeting major adipogenic regulators<sup>12-14</sup>. miRNAs are closely linked to metabolic diseases like T2D and obesity<sup>15,16</sup>. Altered expression of miRNAs in AT, pancreas and liver have shown to be associated with obesity and

metabolic disorders<sup>17-19</sup>. miRNAs could induce IR in AT by affecting adipocyte function or via stimulating local inflammation. Several obesity-related WAT miRNAs like miR-25, -93, -106b family and miR-221 have shown positive correlation with insulin resistant clinical parameters<sup>20,21</sup>. miR-221 is negatively associated with mRNA coding tumour necrosis factor alpha (TNF- $\alpha$ ) and directly targets ADIPOR1, which could contribute to the progression of IR<sup>21-23</sup>. miR-192 has been previously associated with liver disease, T2D and cancers<sup>24-26</sup>.

ANGPTL8 is a family member of ANGPTL protein family. It is highly expressed in both adipocyte and liver and plays an important role in TG trafficking. It is highly induced upon refeeding, insulin treatment and acts as an inhibitor of lipoprotein lipase (LPL) in concert with ANGPTL3<sup>27-29</sup>. siRNA-mediated knockdown of ANGPTL8 causes a reduced TG content in the AT of mice<sup>27</sup>. Recent studies have reported elevated plasma levels of ANGPTL8 in obesity, IR and T2D<sup>30-32</sup>. ANGPTL8 was found to be highly expressed upon insulin treatment *in vivo* in human WAT. Indicating a possible independent intracellular role in adipocytes<sup>33,34</sup>. Despite major advances in ANGPTL8 research, its intracellular function in adipocytes is not well understood.

The rise in prevalence of obesity has gathered interest in the biology of WAT. A comprehensive understanding of the cellular and molecular regulation underlying obesity, IR and T2D is crucial for the development of new therapeutic interventions.

## **2. REVIEW OF THE LITERATURE**

### **2.1 Adipocyte biology**

#### **2.1.1 Adipocyte function**

##### *2.1.1.1 Adipose tissue*

Adipose tissue is a complex organ which regulates and maintains whole body energy homeostasis, along with endocrine functions. Adipose tissue accounts for 20% of the total body weight in a healthy human being with BMI 25<sup>35</sup>. Adipose tissue in mammals is classified into two subtypes: White adipose tissue (WAT) and brown adipose tissue (BAT). Brown adipose tissue differs from WAT by the presence of characteristic adipocytes with multiple smaller lipid droplets and relatively high number of mitochondria. BAT dissipates heat by oxidising stored TGs through highly expressed uncoupling protein 1 (UCP1)<sup>36</sup>. WAT is characterized by the presence of mature adipocytes with huge unilocular lipid droplets which account for more than 90% of the tissue mass. Along with mature adipocytes, WAT contains pre-adipocytes and adipocyte precursor cells and other cell types including endothelial cells, fibroblasts, macrophages and leucocytes. WAT is further subdivided into two categories based on the stereotypical distribution: Visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). Regardless of their histological resemblances VAT and SAT are reported to have different depot-specific metabolic roles<sup>2</sup>. The perception of adipocyte biology two decades ago was limited to its conventional function as fat repository to provide mechanical support and thermal insulation. Discovery of leptin in the year 1994<sup>37</sup> uncovered adipose tissue as a functional endocrine organ and gave insights to the critical roles of AT in human physiology.



### 2.1.1.2 Energy storage

Adipocytes are evolved to store surplus amount of energy in the form of neutral TGs. These TGs can hold more energy per unit mass than protein, carbohydrate or glycogen. Oxidation of one gram fat can yield 9 kilocalories (kcal) of energy whereas carbohydrates and proteins can only release 4 kcal/gram<sup>38</sup>. TGs are nonpolar in nature and can be stored safely in adipocytes in anhydrous form to prevent lipotoxicity to neighbouring tissue<sup>39</sup>. As an energy storing entity, adipocytes efficiently store TGs after feeding and secrete FFAs during starvation. The complex bidirectional nutrient storage and mobilization are regulated by two important biological processes called lipogenesis and lipolysis.

### 2.1.1.3 Triglyceride metabolism

Dietary fat is the main source of FFAs for TG synthesis. Fat is digested in the gastrointestinal tract by the action of pancreatic hydrolytic enzymes to yields FFAs. Epithelial cells of small intestine absorb FFAs, re-esterify then to TGs and integrate them into chylomicrons. TG containing chylomicrons enter portal circulation via the lymphatic system. Lipoprotein lipase (LPL) in the adipose tissue hydrolyses TGs to release FFAs from circulating lipoproteins and facilitates their entry into adipocytes<sup>40</sup>. LPL is critical in lipoprotein metabolism. Apart from adipose tissue, it is highly expressed in liver, muscle and heart tissue. LPL activity is carefully coordinated in a tissue-specific manner to meet varying energy needs. LPL is a rate-limiting enzyme regulated by post-translational mechanisms which include protein-protein interactions with apolipoproteins and members of angiopoietin like protein family (ANGPTL)<sup>40</sup>. FFAs taken up by adipocytes from circulation are re-esterified to TG. *De novo* lipogenesis (DNL) is a concurrent process involving fatty acid synthesis from acetyl coenzyme A (acetyl-CoA) and subsequent biosynthesis of TG. Glucose entry to plasma stimulates the secretion of insulin from pancreas. Insulin facilitates the entry of glucose into adipocytes via GLUT1 and GLUT4 and further activates various glycolytic and lipogenic signalling cascades and triggers the expression of

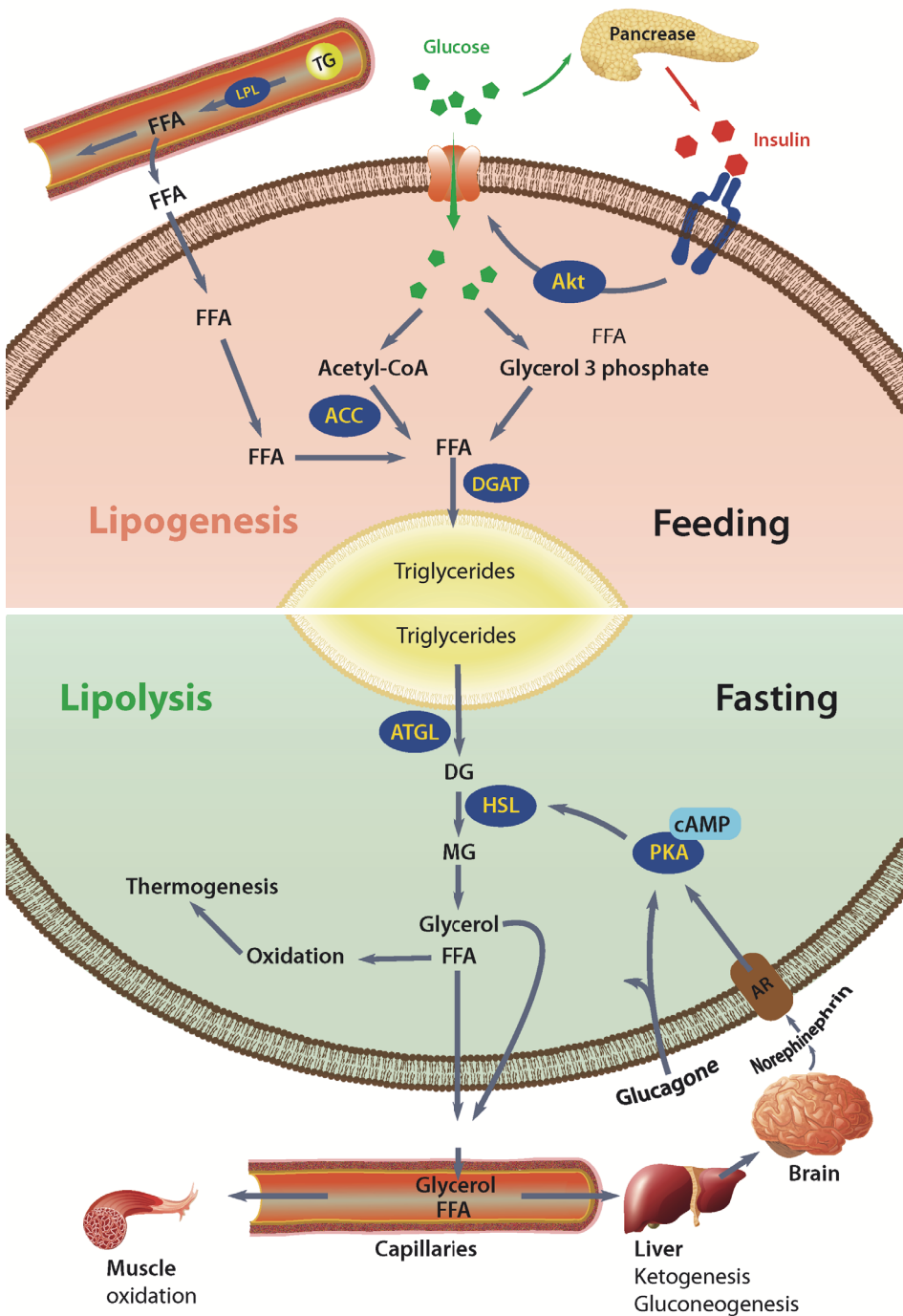
sterol regulatory element-binding protein 1 (SREBP1) which is a master regulator of TG synthesis<sup>41</sup>. Acetyl-CoA and malonyl-CoA derived from glucose are used as substrates to generate palmitate in a crucial step of DNL catalysed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Diacylglycerol acyltransferase (DAG) catalyses the final and only committed step of the sequential esterification process to yield TG<sup>42</sup>.

During energy deprivation adipocytes undertakes a shift towards lipolysis where stored TGs in lipid droplets (LDs) are hydrolysed to FFAs and glycerol to be used as energy source by other tissues. Lipolysis is highly regulated through hormonal and biochemical cues. Catecholamine and glucagon are primary inducers of lipolysis. Catecholamine initiates lipolysis by binding beta-adrenergic receptors on plasma membrane. These receptors coupled with Gs-proteins stimulate adenylyl cyclase to produce cyclic AMP (cAMP). Protein kinase A (PKA) is activated upon cAMP binding<sup>43</sup> and it further phosphorylates hormone sensitive lipase (HSL) and perilipin (PLIN). Upon phosphorylation, HSL is translocated from cytosol to the LD surface<sup>44-47</sup>. In a parallel action, PLIN facilitates LD remodelling to provide more surface area and enhances the activity of HSL on the surface of LDs<sup>48,49</sup>. TGs are then sequentially hydrolysed to diacyl glycerol (DAG) and monoacyl glycerol (MAG) releasing one FA in each step to finally yield glycerol. The enzymatic process is catalysed by adipocyte triglyceride lipase (ATGL), HSL and monoacyl-glycerol lipase respectively<sup>50</sup>. Hydrolysed FFAs are oxidized within the AT or released into portal circulation and taken up by other organs as energy substrate. Glycerol on the other hand is transported to the liver for gluconeogenesis<sup>51</sup>.

#### *2.1.1.4 Endocrine function*

White adipose tissue is a major endocrine organ secreting a wide range of bioactive molecules termed adipokines<sup>37,52</sup> which regulate systemic metabolic functions and regulate crucial biological processes like insulin sensitivity, immune response, blood pressure, bone mass, thyroid function, haemostasis and reproductive function<sup>53</sup>. Recent studies have reported a growing number of secretory molecules and their relevance for

the various physiological functions of adipose tissue<sup>54</sup>. Discovery of leptin in 1994<sup>55</sup> and cloning of adiponectin in 1995<sup>56</sup> were major breakthroughs. Leptin is a satiety hormone secreted by white adipocytes targeted to hypothalamus where it regulates energy balance by inhibiting appetite<sup>37,57,58</sup>. Apart from its satiety role, leptin has also been shown to regulate reproductive organs<sup>59,60</sup>. Some evidence show that it modulates glucose homeostasis in the  $\beta$ -cells of pancreas by regulating expression and secretion of insulin<sup>61</sup>. Adiponectin is one of the important adipokine synthesised and secreted from adipocytes in relatively high concentrations ( $\sim 10$ -30  $\mu\text{g/ml}$  or about 0.01% of plasma proteins)<sup>62</sup>. It is well known for its insulin sensitizing, anti-inflammatory and cardiovascular protective effects<sup>63-67</sup>. Adiponectin primarily targets liver, activates AMP activated protein kinase to negatively regulate gluconeogenesis<sup>68,69</sup> and increases hepatic insulin sensitivity independent of AMPK by upregulating hepatic ceramidase activity<sup>70</sup>. Apart from the liver, adiponectin targets skeletal muscle where it has been shown to increase the phosphorylation of ACC, glucose uptake and fatty acid oxidation by activating (AMPK) pathway<sup>63,71</sup>. Many other adipokines like resistin and chemerin are thought be of adipocyte origin. However, recent evidence has suggested that they are expressed in different cell types within adipose tissue and exert pleiotropic functions<sup>72-74</sup>



**Figure 1. Lipogenesis and lipolysis.** Lipid metabolism and distribution controlled by adipose tissue. Lipogenesis is a process by which carbohydrates are converted into fatty acids and become a substrate for the biosynthesis of TG in adipocytes. Lipolysis works in the reverse way, breaking down TG to free fatty acids (FFAs) and glycerol which are either oxidized or secreted. The absorption of circulating FFAs by liver, muscle and other tissues is a primary process of lipid sorting. Both lipogenic and lipolytic pathways respond to nutrition and hormones such as insulin, norepinephrine and glucagon. Hence, a refined regulation of lipogenesis and lipolysis is essential for energy homeostasis and insulin sensitivity. cAMP, cyclic adenosine monophosphate; AR, adrenergic receptor; PKA, protein kinase A; IR, insulin receptor. This figure is modified from Luo, L. Liu, M., *The Journal of Endocrinology*; 2016; 231(3): R77-R99<sup>75</sup>.

## 2.1.2 Role of adipose tissue in metabolic pathophysiology

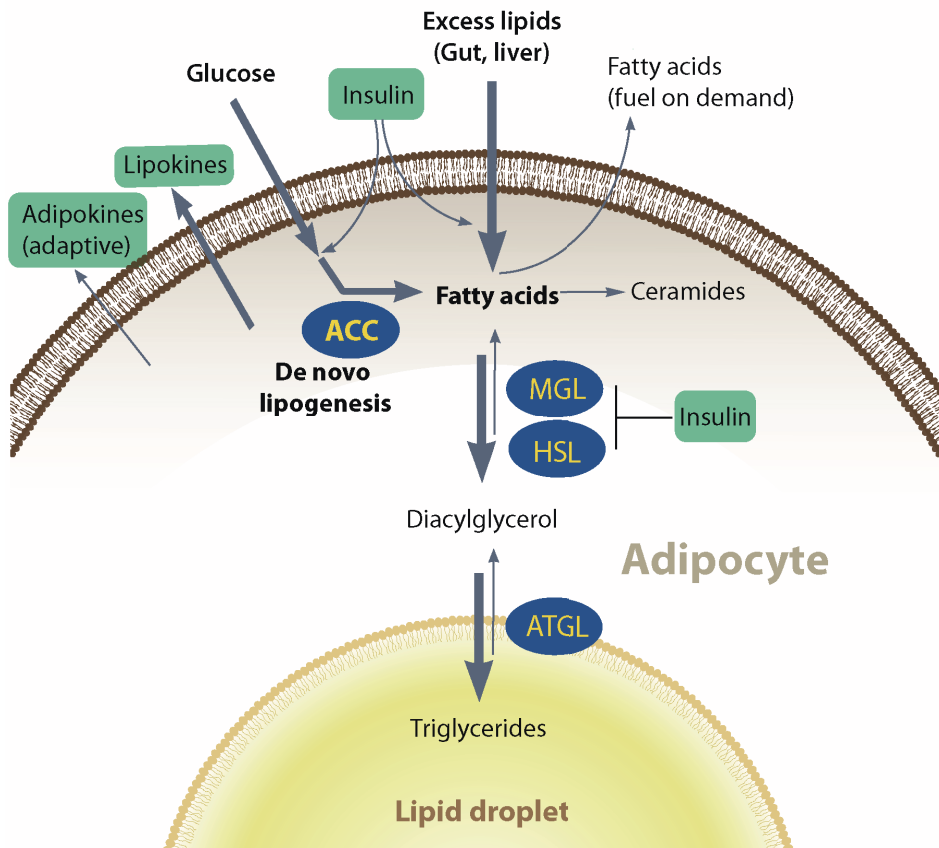
### *2.1.2.1 Adipose tissue distribution and turnover*

Dysfunction of adipose tissue is strongly associated with severe metabolic complexities like obesity, T2D, metabolic syndrome and lipodystrophy. WAT is systematically distributed across the body. Specific location of WAT defines its identity and primary function. In humans, accumulation of intra-abdominal WAT or visceral adipose tissue (VAT) is associated with obesity-linked comorbidities. AT from this depot is highly metabolically active and dysregulation significantly increases the risks for metabolic disorders<sup>76</sup>. On the other hand, SAT relatively poses less risk and has been reported to exert protective metabolic functions<sup>4</sup>. Reason behind differences in fat distribution among individuals remains to be addressed. However, several factors like genetic predisposition<sup>77</sup>, sex hormones<sup>78</sup>, usage of glucocorticoids<sup>79</sup> and epigenetics<sup>80,81</sup> play a vital role in determining the location of excess fat derived from over feeding. Adipocyte turnover is a continuous process which relies on the balance between adipogenesis and apoptosis. Total adipocyte number is determined during childhood and adolescence and remains constant through life. AT relies on adipocyte progenitor cells from stromal vascular fraction (SVF) as a source for regeneration. Average lifespan of an

adipocyte in an adult is approximately 10 years with 10% of SAT replaced annually<sup>82</sup>.

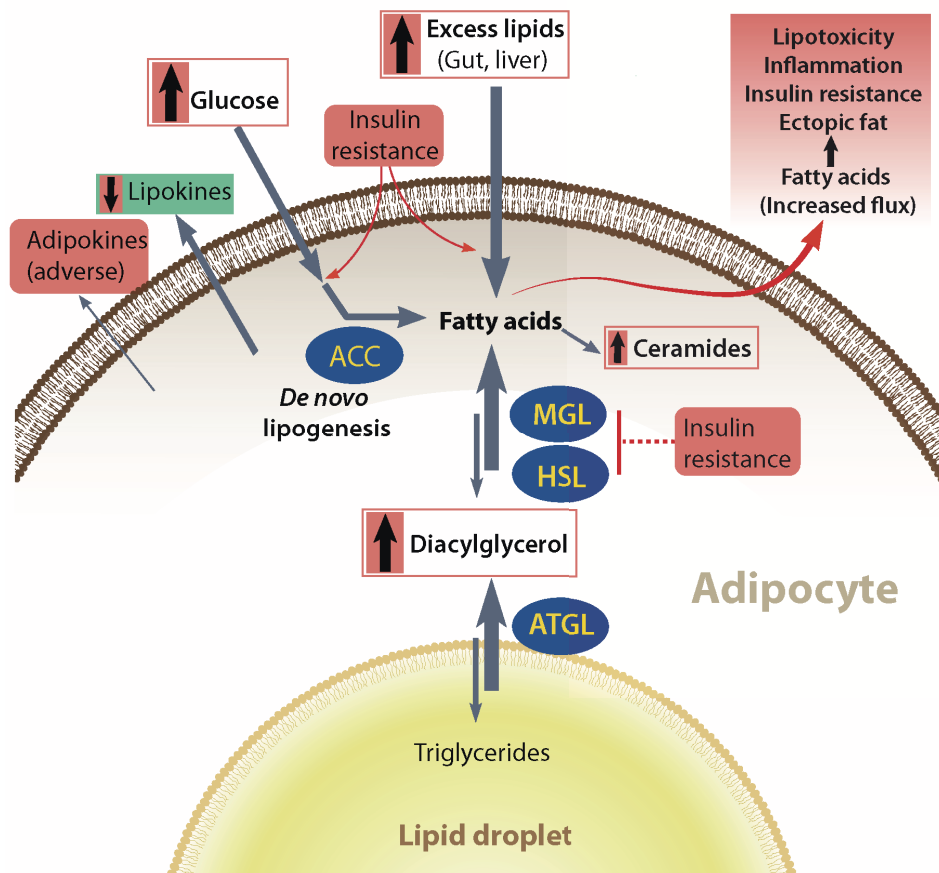
#### *2.1.2.2 Altered lipid mobilization, sorting and storage*

Efficient FA uptake in postprandial state is a very important function of adipocytes to avoid adverse systemic effects of high plasma FA concentrations<sup>83</sup>. FAs are important physical components of plasma membrane lipids and active energy substrates. They play roles as second messengers and are implicated in skeletal and hepatic tissue insulin resistance<sup>84</sup>. Now it is well documented that AT storage capability is crucial to avoid FA- based lipotoxicity in target cells<sup>85</sup>. However, AT lipid buffering capacity is impaired in obesity due to inability to respond to postprandial state and in lipodystrophy due to the lack of AT<sup>83</sup>. FA mobilization after TG lipolysis characterizes the other section of FA homeostasis. It is well documented that disturbed lipolysis interrupts the bioavailability of FAs. LPL plays a major role in regulating intravascular and lipolysis. Various LPL mutations abolish production of the LPL transcript or lead to unstable transcripts. Some missense mutations prevent secretion of LPL from cells, others interfere with the formation of unstable or catalytically active homodimers<sup>86</sup>. Loss of LPL activity causes familial chylomicronaemia syndrome, marked by elevated levels of plasma TG<sup>87</sup>. After crossing the endothelial barrier, several FA binding proteins facilitate FA incorporation into TG and LD-associated proteins efficiently package TG to LD. Hence LPL activity and normal TG synthesis and efficient fat storage pathways are crucial to avoid FA spill over which eventually leads to ectopic fat storage.



**Figure 2. Lipid metabolism and storage in normal and dysfunctional adipocytes.**

A. Healthy adipocytes store and metabolize surplus circulating lipids and glucose in the inert form of TG in the lipid droplets. Fatty acids can be trafficked via lipolysis when required. Pancreatic insulin initiates *de novo* lipogenesis as well as the absorption and storage of circulating lipids. The secretion of adipokines by the adipocytes contributes to systemic metabolic regulation.



**2 B.** Abnormal TG storage in hypertrophic or dysfunctional adipocytes is connected with basic fatty acid mobilization, lowered glucose uptake and *de novo* lipogenesis, and lipotoxic diacylglycerols and ceramides are accumulated in distant tissues. Changes in these metabolic states are partly due to IR. Dysfunctional adipocytes are characterized by lowered production of some lipokines as well as altered adipokine profile. Taken together, the increased flux of FAs from adipocytes induces systemic metabolic dysfunction. Adaptive and adverse effects are marked in green and red, respectively. HSL, hormone-sensitive lipase; ATGL, adipose triglyceride lipase; MGL, monoglyceride lipase; ACC, acetyl-CoA carboxylase. This figure is adapted and modified from Vegipoulos et al., EMBO Journal: 2017 Jul 14;36(14):1999-2017



### *2.1.2.3 Adipocyte hypertrophy and hyperplasia*

Adipocyte hypertrophy is characterized by the presence of large lipid-laden adipocytes due to expansion in cell size beyond 800 pL, the normal volume range. On the other hand, hyperplasia is a process where relatively smaller adipocytes are newly differentiated from the preadipocyte pool of SVF<sup>88</sup>. Both adipocyte hypertrophy and hyperplasia are important characteristic features of AT expansion upon nutrient influx<sup>82,88</sup>. Prolonged positive energy balance, genetic predisposition and various environmental factors contribute to expansion of AT<sup>89</sup>. The risk of developing insulin resistance (IR) and T2D goes hand in hand with the increased adipose tissue mass and fat deposition<sup>90</sup>. There is an inverse quantitative relation between lower rate of adipocyte production and higher grade of hypertrophy. Subjects with hypertrophy are shown to generate 70% less adipocytes per year compared to those with hyperplasia<sup>91</sup>. Enlarged adipocyte volume correlates with serum insulin levels, insulin resistance and elevated risk of developing T2D<sup>92</sup>. Hypertrophy is associated with AT inflammation, AT fibrosis and ectopic fat deposition<sup>82,93-95</sup>. Hypertrophy initiates cellular stress and activates Jun N- terminal kinase (JNK), nuclear factor-kB (NF-kB), Mkk4 and other stress inducible master regulators<sup>96,97</sup> which activate the transcriptional and protein phosphorylation cascade leading to an abrupt release of pro inflammatory secretome from adipose tissue which consists of pro-inflammatory cytokines and chemotactic signalling molecules like IL-6, TNF- $\alpha$ , interleukin 1 beta (IL1 $\beta$ ), monocyte chemoattractant protein 1 (MCP1) and others. This condition further leads to local and systemic inflammation<sup>98</sup>. Hypertrophic adipocyte expansion may lead to hypoxic condition causing elevated levels of hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) initiating AT fibrosis in association with local AT inflammation<sup>99</sup>. Hyperplasia, on the other hand, is generally considered healthy expansion of AT where new relatively smaller adipocytes are formed. A typical hyperplastic expansion of AT maintains an anti-inflammatory state with elevated levels of regulatory T cells, M2 AT macrophages, and exhibits high insulin sensitivity and adequate vasculature for expansion<sup>100</sup>.

#### *2.1.2.3 Lipodystrophy*

Lack or entire absence of metabolically active adipose tissue defines lipodystrophy. Intensity of lipodystrophy can be determined by the degree of AT deficiency, which can be acquired or originates due to genetic abnormalities<sup>101</sup>. AT deficiency leads to ectopic deposition of TG in liver or skeletal muscle causing reduced whole-body insulin sensitivity and organ dysfunction<sup>102,103</sup>. Defective AT storage elevates circulating levels of TG, cholesterol and other metabolically active lipid species, leading to atherosclerosis and CVD, as reported in AT deficient Seipin knockout mice<sup>104</sup> and in patients suffering from congenital generalized lipodystrophy<sup>105</sup>. In addition to lipid storage defects, adipokine and cytokine levels are generally altered in lipodystrophy. Reduced levels of both adiponectin and leptin are reported in generalized lipodystrophy<sup>106</sup>.

#### *2.1.2.4 Adipocyte Inflammation*

Adipose tissue has emerged as a biologically active organ linking metabolic, endocrine and immune functions to maintain whole body homeostasis. A wide range of immune cells are abundantly present in adipose tissue stromal vascular fraction which makes them the second most abundant cell type after mature adipocytes. Immune cells residing in adipose tissue play an important housekeeping role. They clear detritus and apoptotic cells to maintain adipose tissue homeostasis in non-obese conditions<sup>107</sup>. However, excess fat accumulation alters the immune cell pools in adipose tissue by increasing macrophages, neutrophils, mast cells, B and T lymphocyte activity and populations<sup>108</sup>. Unlike normal inflammatory response which is triggered by an infection, adipose tissue inflammation is associated with a chronic low grade sterile inflammation where slight increase in circulatory pro-inflammatory factors are seen which might not be clinically evident<sup>109</sup>. Despite low severity, inflammation caused by obesity can exert substantial effects on metabolic pathways which may lead to IR<sup>110,111</sup>. Secretion of pro-inflammatory molecules from dysfunctional adipocytes initiates the migration of monocytes from systemic circulation to AT where they eventually differentiate into macrophages<sup>96</sup>. These infiltrated cells in turn enhance

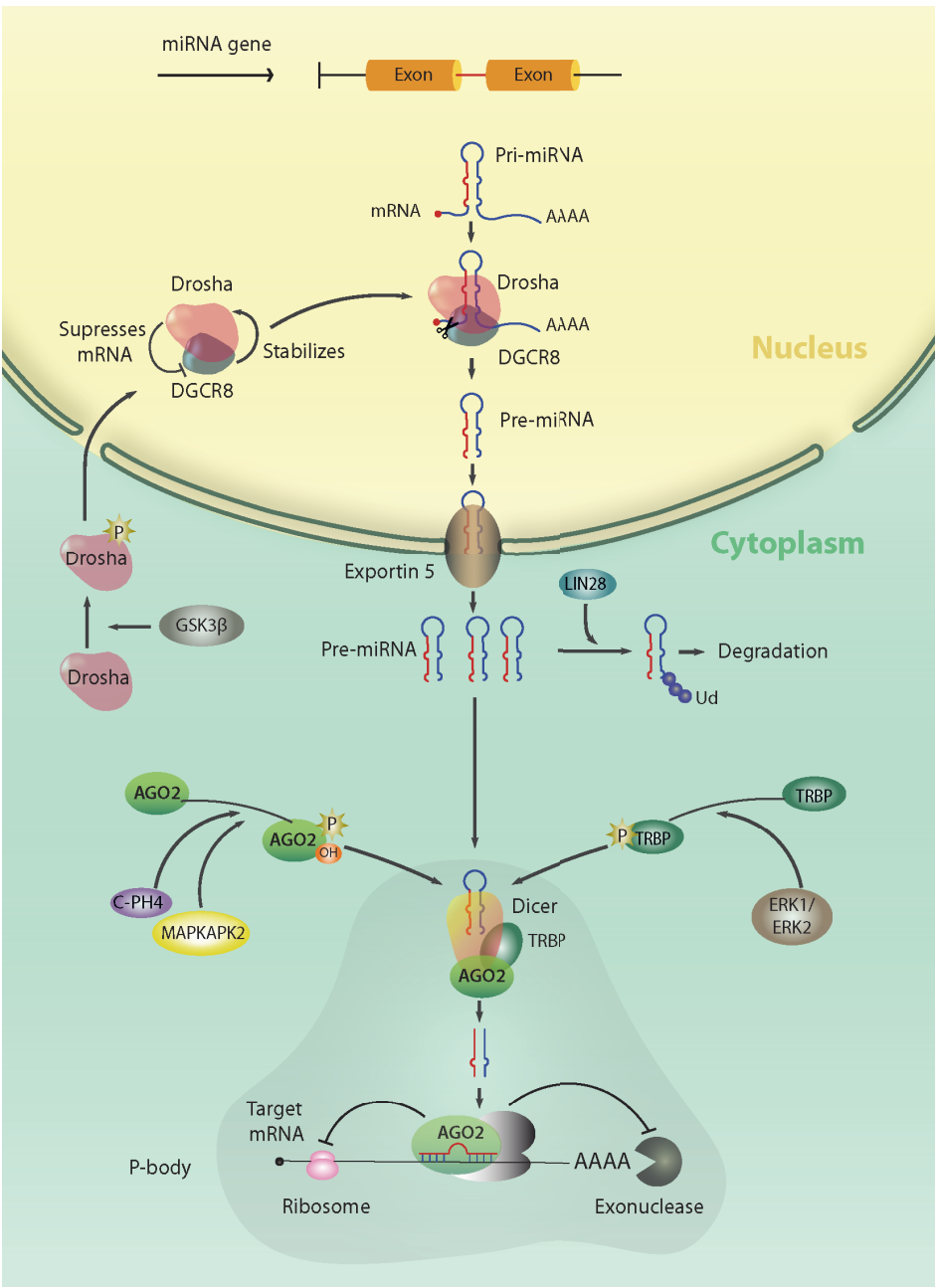
the inflammatory status by secreting similar pro-inflammatory molecules resulting in both local and systemic inflammation<sup>98</sup>.

## 2.2 MicroRNAs

MicroRNAs(miRNA) are noncoding 22 nucleotides long small RNA molecules of endogenous origin systematically evolved in eukaryotes to cut down the redundant genetic transcripts<sup>112-115</sup>. miRNAs regulate diverse cellular processes including cell proliferation, differentiation and survival by specifically targeting complementary mRNA transcripts causing either degradation or translation repression<sup>113,116-119</sup>. The first miRNA lin-4 was discovered in *Caenorhabditis elegans* in 1993<sup>113</sup> and the first mammalian miRNA let-7 was identified seven years later<sup>114</sup>. These two studies initiated genomic research which lead to the identification of many miRNA and noncoding RNA species<sup>113,114,120-122</sup>. Functional validation of these miRNAs has led to an improved understanding of cell biology at the molecular level.

### 2.2.1 MicroRNA biogenesis

Biogenesis of miRNAs in humans is highly regulated by four important enzymes Drosha, Exportin 5, Dicer and Argonaut 2 (AGO2) described in Fig 3. A majority of the miRNA encoding genes are located in intronic regions with their own promotor segments. miRNA is transcribed by RNA polymerase II to give rise to a long (around 1kb) primary miRNA (pri-miRNA) transcript<sup>120,123</sup>. A typical pri-miRNA consists of 33-39 nucleotide long hairpin stem<sup>124</sup> and single stranded RNA overhangs at both 5' and 3' end. The first step of the maturation process is initiated in the nucleus. A microprocessor complex containing Drosha (RNAs III endonuclease) processes the pri-miRNA and DiGeorge syndrome critical region 8 (DGCR8), a cofactor, helps in RNA binding<sup>125</sup> and recognizes cutting sites. Drosha cuts the region spanning the stem-loop of pri-miRNA releasing a hairpin shaped RNA of ~65 nucleotides in length (pre-miRNA)<sup>126</sup>. A nuclear transport complex of Exportin 5 and GTP binding



**Figure 3. MicroRNA biogenesis.** Biogenesis of microRNAs (miRNAs) generally with transcription of miRNA parent gene by RNA polymerase II to create a primary miRNA (pri-miRNA) hairpin, which is then taken up by the Drosha-DGCR8 (DiGeorge syndrome critical region 8) complex which gives rise to precursor miRNAs (pre-miRNAs). Exportin 5 transports these molecules into the cytoplasm, where they further undergo cleavage by Dicer-TRBP (TAR RNA-binding protein 2) and are loaded into Argonaute 2 (AGO2)-containing RNA-induced silencing complexes (RISCs) to dampen downstream target gene expression. This figure is adapted and modified from Li Z, Rana TM., *Nat Rev Drug Discov.* 2014 Aug;13(8):622-38

Nuclear protein RAN-GTP together transport the pre-miRNA to cytoplasm in a GTP-dependent manner<sup>127-129</sup>. In cytoplasm, Dicer (RNAs III type endonuclease) and TAR RNA binding protein (TRBP) bind the pre-miRNA and Dicer cleaves near the terminal loop releasing a ~22 nucleotide miRNA duplex<sup>130,131</sup>. This RNA duplex has two potential mature miRNA strands, miRNA in 5' and miR\* or miR-3p in the 3' end<sup>132</sup>. miR\*/3p originating from the 3' strand was earlier reported to be less frequently expressed. However, strand selection is not obligatory as alternate (miR\*) strand selection has been reported in miRNA isoform sequencing study<sup>133</sup>. The mechanism for strand selection by RNA-induced silencing complex (RISC) during pri-miRNA processing could be partially explained by the model where (model 1) similar thermodynamic features of ds-miRNA duplex termini may lead to selection of both strands and (model 2) in the case of strands with dissimilar thermodynamic property, strand with relatively higher thermodynamic stability is favoured for processing<sup>134,135</sup>. In the following step, RNA duplex is successively loaded to (RISC). miRNA processing in RISC is mediated by argonaute protein (AGO) family of proteins along with many cofactors like TRBP (or PACT). After miRNA duplex loading, pre-RISC complex along with Ago proteins cleaves the passenger strand to give rise to mature miRNA.

## 2.2.2 MicroRNA function

After processing, miRNA is successfully loaded into miRNA induced silencing complexes (miR-RISC). Argonaute family proteins (AGO) serve as a major function unit of miR-RISC. In mammals, there are four AGO proteins AGO 1-4, which mediate mRNA repression. AGO2 is the only member of the AGO family that functions in RNAi causing mRNA degradation<sup>136-138</sup>. Through RISC complex, miRNA executes two ways of gene silencing, mRNA degradation and translational repression<sup>139</sup>. In animals, miRNAs recognize and partially bind target mRNAs via their seed sequence between nucleotides 2-8<sup>139,140</sup>. Following target recognition, miRNA induces deadenylation via GW182 which recruits effector proteins like poly(A)-binding protein (PABP) and additional deadenylase complexes, PAN2-PAN3 and CCR4-NOT complexes<sup>141-143</sup>. After deadenylation, decapping inducers including DDX6 are assembled onto the CCR4-NOT complex, initiating decapping by DCP2. In the final step, XRN1 exonucleolytically cleaves the mRNA from 5'-3'<sup>144,145</sup>. Three general mechanisms have been suggested for miRNA mediated translation repression. (i) Repression at initiation of translation: GW182 dependent PABP dissociation from poly-(A) tail disrupts the structure formed by the interaction between eIF4G and PABP. mRNAs without poly(A) tail are also repressed by miRNA or GW182 tethering<sup>146,147</sup>. (ii) GW182 can also exert translational repression by recruiting CCR4-NOT translation inhibitor complex<sup>148</sup>. (iii) miRNAs can dislodge eIF4 from target mRNA which in turn inhibits ribosome binding<sup>149,150</sup>. The mechanisms mentioned above are not completely independent. They may occur alongside, overlap with each other, or take place with different kinetics to enhance the comprehensive silencing effect.

## 2.2.3 MicroRNAs in adipocyte biology

### 2.2.3.1 MicroRNAs in adipocyte differentiation

miRNAs play a central role during the fate of adipocytes. Increasing number of studies carried out *in vivo* and *in vitro* in both human and

mouse models have reported the integral role of miRNAs during adipocyte formation and function. Inhibition of Drosha and Dicer, key proteins of miRNA biogenesis have been reported to inhibit adipocyte differentiation in human mesenchymal stem cells and inhibition of Drosha in 3T3-L1 has been shown to block adipogenesis<sup>151,152</sup>. miR-143 is the first miRNA reported to be involved in human adipogenesis. Interestingly, the effect of its direct target ERK5 on adipocyte formation was not known before<sup>8</sup>. Direct targeting and regulation of the master regulator of adipogenesis, PPAR gamma by miR-27b was identified in 2009<sup>153</sup>. Several anti-adipogenic miRNAs include miR-130 which directly targets PPAR gamma<sup>13</sup>, miR-138 which targets EID-1, a cofactor which binds to PPAR gamma to enhance its transcriptional activity<sup>154</sup> and miR-375, which directly targets and represses AdipoR2<sup>155</sup>. Furthermore, many pro-adipogenic miRNAs have been identified. miR-30c facilitates the regulation of adipokines: it represses two targets, PAI-1 and ALK2, from different signalling pathways<sup>9</sup>. The combined action of miR-17-5p and miR-106 has been shown to regulate the balance between osteogenic and adipogenic differentiation by targeting BMP2<sup>156</sup>. miR-26a and miR-26b are upregulated in early adipocyte differentiation. They target sheddase ADAM metalloprotease domain 17 (ADM17/TACE), which upregulates UCP1 expression<sup>11</sup>. A well-known promoter of adipogenesis, miR-148a, directly targets WNT1, which is a well-known inhibitor of adipogenesis<sup>157</sup>.

#### 2.2.3.2. *MicroRNAs in obesity*

Numerous studies have profiled miRNA expression levels between obese versus lean AT in both mice and humans. In a diet-induced obese mice study, out of 574 detected miRNAs, only 35 miRNAs were found differentially expressed<sup>158</sup>. Moreover, several miRNA profiling studies in human WAT have identified miRNAs which might be dysregulated in patients with obesity along with or without T2D. Nevertheless, further studies with *in vitro* experimental validation in different adipocyte cell systems have revealed miRNAs involved in obesity (Table 1). Apart from overfeeding, genetics plays an important role in obesity. However, in human WAT, the degree of differential expression of miRNAs is

significantly smaller compared to the relative expression of mRNAs<sup>159</sup>, and altered miRNA levels associated with obesity in humans positively correlate with those in the WAT of obese mice affected by genetics or high fat diet<sup>158</sup>. Some of the miRNAs differentially expressed in human WAT are summarized in Table 1. Discrepancies are found in the miRNA expression in different adipocyte depots. In miRNA expression profiling from omental and subcutaneous WAT of either overweight or obese subjects, 16 miRNA transcripts out of 106 showed depot specific expression and high expression levels in omental compared to subcutaneous WAT<sup>15</sup>. Further studies are needed to identify more miRNAs differentially expressed in human WAT and their possible role in obesity.

**Table 1. miRNAs dysregulated in obesity**

miRNA	Species and/or depot	Expression status	Phenotype and or function	Study
<b>miR-21</b>	Human/subcutaneous	Upregulated ↑	Not established	171
<b>miR-130</b>	Human/subcutaneous	Upregulated ↑	Not established	13
<b>miR-146b</b>	Human/subcutaneous and visceral	Upregulated ↑	Not established	159
<b>miR-222</b>	Human/visceral	Upregulated ↑	Not established	169
<b>miR-324-3p</b>	Human/ AT/MCS	Upregulated ↑	Induces adipogenesis	163
<b>miR-519d</b>	Human/subcutaneous	Upregulated ↑	Induces PPAR-alpha	160
<b>miR-221</b>	Human/subcutaneous	Up/down ↑↓	Targets ADIPOR1, EST1	21,165
<b>Let7a/d</b>	Human/subcutaneous	Down ↓	Not established	166
<b>miR-17-5</b>	Human/visceral	Down ↓	Not established	170



<b>miR-26a</b>	Human/subcutaneous	Down	↓	Associated with lipolysis, secretion of CCL2 and TNF $\alpha$	166,167
<b>miR-29-b</b>	Human/subcutaneous	Down	↓	Associated with TNF $\alpha$ expression	168
<b>miR-125a</b>	Human/visceral	Down	↓	Not established	173
<b>miR-126</b>	Human/subcutaneous	Down	↓	Inhibits CCL2	166
<b>miR-132</b>	Human/visceral	Down	↓	Regulates immune system	170
<b>miR-141</b>	Human/visceral	Down	↓	Targets YWHAG	164
<b>miR-143</b>	Human/subcutaneous	Down	↓	Not established	171
<b>miR-150</b>	Human/subcutaneous	Down	↓	Not established	160
<b>miR193b</b>	Human/subcutaneous	Down	↓	CCL2 secretion	166
<b>miR-200b</b>	Human/visceral	Down	↓	Not established	172
<b>miR-220a</b>	Human/visceral	Down	↓	Not established	172
<b>miR-520</b>	Human/visceral	Down	↓	Targets RAB11A	164
<b>miR-1275</b>	Human/visceral	Down	↓	Targets ELK-1	161,162

This table is adapted and summarized from Arner et al<sup>160</sup>, Brandao et al<sup>161</sup>, JA Deuliis<sup>162</sup>.

### 2.2.3.3 MicroRNAs in adipose tissue inflammation

Many miRNAs have been reported to regulate AT inflammation. miR-132, which is known to be downregulated in visceral WAT in obese subjects<sup>163</sup>, triggers NF- $\kappa$ B of B- cells initiating transcription of IL-8 and CCL2 (a key factor involved in obesity-induced inflammation) in mature adipocytes *in vitro* and in human primary adipocytes<sup>164</sup>. Several miRNAs which are

otherwise expressed equally in WAT are altered in obesity and have been reported to regulate human WAT inflammation by reducing CCL2 secretion from macrophages and adipocytes<sup>165</sup>. miRNA-223 knockout mice, when fed with high fat diet, developed severe systemic insulin resistance and AT inflammation. miR-223 directly targets an important macrophage polarizing factor Pknox1 which reduces diet-induced AT inflammation<sup>166</sup>. Reduced expression levels of miR-221, a family member of miR-221-3p, one of the main targets of this study, negatively correlated with TNF- $\alpha$  in mature adipocytes originated from mesenchymal stem cells of obese female subjects<sup>23</sup>. Many more miRNAs directly or indirectly regulate AT inflammation either by inducing the secretion of adipokines and or regulating macrophage activation<sup>160</sup>.

#### *2.2.3.4 Role of microRNAs in insulin resistance (IR) and T2D*

Insulin resistance is the main pathological condition tightly associated with T2D. In this condition the target cells lose the ability to respond to insulin stimulation. T2D is a complex disorder which is characterized by impaired glucose tolerance, defect in  $\beta$  cell function and hyperglycaemia. In recent years, many studies have reported the involvement of miRNAs in  $\beta$  cell development, insulin secretion and insulin signalling cascade related to IR. Several well studied miRNAs like miR-7, miR-9, miR-15a/b, miR-34a, miR-124a, miR-195 and mi-376 have been reported to regulate glucose homeostasis and insulin production<sup>167,168</sup>. Insulin growth factors (IGF1 and IGF2) play an important role in regulating IR and T2D<sup>169,170</sup>. miR-1 has been reported to regulates the expression of IGF-1-R1<sup>171</sup> and IGF-1<sup>172</sup>. miR-320 has been shown to regulate the expression of IGF-1 in endothelial cells and to regulate IR in 3T3L-1cells<sup>173</sup>. Study by Agarwal et al. describes that the miR-135a markedly decreases the IRS-2 protein expression and miR-135a knock-down has been shown to increase the expression of both IRS-2 transcript and protein expression<sup>174</sup>. miRNAs miR-320<sup>173</sup>, miR-126<sup>175</sup> and miR-29<sup>176</sup> have been documented to regulate PI3K subunit expression which may ultimately cause IR. GLUT4, a major glucose transporter is also regulated by number of miRNAs leading to lowered glucose uptake. miR-133 has been reported to regulate GLUT4

expression via targeting KLF15 in cardiomyocytes<sup>177</sup>. Interestingly, reduced expression of miR-21 was seen in insulin resistant adipocytes and the same study showed that over expression of miR-21 led to insulin-stimulated translocation of GLUT4 in insulin resistant 3T3L1 adipocytes<sup>178</sup>.

#### *2.2.3.5 Circulating microRNAs*

Cell-free miRNAs are found distant from their origin in circulation generally embedded within exosomes, HDL and RISC<sup>179,180</sup>. A significant number of circulating miRNAs originate from AT. 7,000 mRNAs and 140 miRNAs have been detected in exosomes secreted from the 3T3-L1 cell line<sup>181</sup>. In a recent study, miRNA expression profile from exosomes of fat specific Dicer KO mice showed a significant decrease of 419 miRNAs out of the 653 detected compared to WT control<sup>182</sup> including several miRNAs that are reported to be highly expressed in AT like miR-16, miR-201, miR-221 and miR-222<sup>22,183,184</sup>. miRNAs might mediate the communication between adipocytes in AT via microvesicles, regulating transcription, TG synthesis, adipocyte growth, size and lipid storage<sup>185-187</sup>. Moreover, AT derived exosomal miRNAs may exert both paracrine and endocrine effects<sup>182</sup>.

#### *2.2.3.6 MicroRNA 192-3p*

miR-192\*/3p is derived from antisense 3' arm of the miR-192 precursor located in chromosome 11-q13.1. Only few studies so far have reported functional characterization of miR-192\*. Expression levels of miR-192\* were depleted upon gliadin peptide treatment in cultured fibroblasts obtained from coeliac disease patients, including upregulation of MMPs via gluten elicit response in coeliac disease fibroblasts<sup>188</sup>. Both miR-192-3p and miR-192-5p are involved in downregulating the farnesoid X receptor (FXR, NR1H4), a ligand-activated transcription factor regulating bile acid homeostasis both in liver and intestine. Especially, miR-192-3p has been shown to target the NR1H4-3' UTR suppressing its expression and its target

genes, OSTa/ $\beta$  and OATP1B3<sup>189</sup>. miR-192-3p has been recently reported to be dysregulated in human rectal cancer tumour samples<sup>190</sup>.

#### *2.2.3.7 MicroRNA 221-3p*

miR-221-3p is a miR-221/222 family member originating from antisense 3' arm of the miR-221 precursor located in chromosome X-p11.3. This family of miRNAs are implicated in many adipocyte metabolic dysfunctions<sup>158,191</sup>. miR-221 has been shown to suppress ADIPOR1 expression, thereby affecting adiponectin signal transduction and insulin sensitivity of adipocytes<sup>192</sup>. miR-222 targets GLUT4 and could affect insulin sensitivity in gestational diabetes<sup>193</sup>. A miRNA screen performed on adipocytes differentiated from human mesenchymal stromal (MCS) cells reported the blunted expression of miR221-3p compared to control (undifferentiated) MCS<sup>194</sup>. Combined interactive analysis between most dysregulated miRNAs and mRNAs has predicted that miR-221-3p might target PGC1- $\alpha$  and phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1)<sup>194</sup>. Elevated expression of miR221-3p has been projected as a potential biomarker for acute myocardial infarction<sup>195</sup>. Dysregulation of miR-221-3p is also reported in various forms of cancer. Overexpression of miR-221-3p along with miR-222-3p and miR106b-25 cluster has been linked with NASH-associated liver carcinogenesis<sup>196</sup>. Moreover, miR-221-3p has been reported to target THBS2, which is an inhibitor of lymphatic metastasis in cervical cancer<sup>197</sup>.

### *2.4 Angiopoietin like 8 (ANGPTL8)*

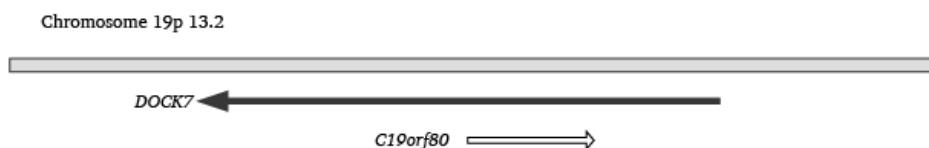
Angiopoietin like 8 is a member of Angiopoietin-like protein (ANGPTLs) family. Till date eight members of ANGPTLs have been discovered (ANGPTL1-8). The nomenclature of ANGPTLs derives from the structural similarities with angiopoietins, which are well known regulators of angiogenesis. Based on the independent findings, ANGPTL8 is also referred as C19orf80, RIFL<sup>27</sup>, Lipasin<sup>28</sup>, betatrophin and hepatocellular carcinoma associated gene TD26. ANGPTL8 is a functional protein expressed in WAT, BAT and liver, and its expression is induced by

feeding, insulin and thyroid hormone. The protein is implicated in various biological processes, mainly lipid metabolism<sup>27-29</sup>, T2D<sup>198</sup> and inflammation<sup>199</sup>.

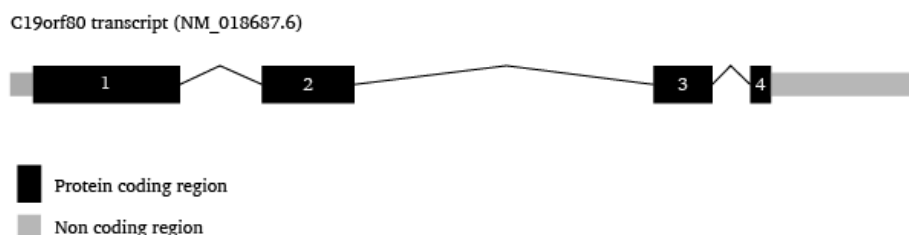
#### 2.4.1 ANGPTL8 Gene structure and protein motifs

Human ANGPTL8 gene (*C19orf80*) is localized on chromosome 19p13.2 embedded in the intron of *DOCK6* and encodes ANGPTL8 mRNA transcript (NM\_018687.6). Mouse ANGPTL8 gene is termed *GM6484* and is located on chromosome 9. Both transcripts span four exons translating 198 amino acid leading to a ~22-kDa protein (Fig. 3) Both human and mouse poly(A) RNA sequence analysis has reported a single ANGPTL8 transcript. Human ANGPTL8 is 73% identical and 82% similar to the murine protein<sup>200</sup>. ANGPTL8 apparently originated due to gene duplication of an inherited *DOCK* gene before evolutionary mammalian radiation. Generally, ANGPTL family proteins share a similar protein domain pattern with a prominent N-terminal domain and a C-terminal fibrinogen-like domain connected through a linker region (Fig. 3). ANGPTL8, unlike its family members, lacks the fibrinogen-like domain at its C terminal and possesses only the coiled-coil domain<sup>29</sup>. Sequence alignment analyses have revealed that the ANGPTL8 N-terminal domain is significantly similar to the N-terminal domains of both ANGPTL3 and ANGPTL4, which are responsible for binding and inhibiting LPL activity<sup>200,201</sup>. Both human and mouse ANGPTL8 include a secretion signal sequence at the N terminus and are predicted to have two coiled-coil domains and a cleavage site between amino acid 15 and 16<sup>202,203</sup>.

A



B



**Figure 3. Gene structure of human ANGPTL8.** A) Gene location of ANGPTL8 on chromosome 19p13.2, B) ANGPTL8 transcript (NM\_018687.6). This figure is adapted and modified from Tseng et al., Int J Mol Sci. 2014 Dec 18;15(12):23640-57

## 2.4.2 Physiological expression

ANGPTL8 is expressed in liver, WAT and BAT of both human and mice. According to Zhang et al, ANGPTL8 is highly expressed in liver and BAT in mice, while in humans, ANGPTL8 is reported to be mainly expressed in liver<sup>200</sup>. Following studies also reported ANGPTL8 expression in mice BAT and its elevation in cold temperature<sup>204</sup>. Parallel expression profile study by reported similar observations in mice but reported a prominent expression also in WAT <sup>27</sup>. ANGPTL8 mRNA expression is reported in both liver and subcutaneous adipose tissue (SAT) of human subjects<sup>34</sup>. Expression analysis from Human Protein Atlas study has documented the enrichment of ANGPTL8 in liver and AT though breast and kidney tissue also express it to some extent<sup>205</sup>.

#### 2.4.2.1. Regulation of expression

Expression of ANGPTL8 is nutritionally regulated. It is highly induced upon refeeding while fasting suppresses its activation<sup>27,200</sup>. Studies from Ren et al. have demonstrated approximately 8 and 12-fold increase in fasted/refed mice WAT and liver, respectively<sup>27</sup>. In several dietary transcriptome studies in mice and human, differential expression of ANGPTL8 could be observed. Microarray analysis in mice showed a decrease of 13% and 6% in ANGPTL8 mRNA expression in the liver after 6 and 12 hrs fasting, respectively<sup>206</sup>. In a dietary manipulation study, human subjects were put under calorie restriction for 8 weeks followed by 2 weeks of high fat diet. During the low-calorie period, ANGPTL8 transcript expression in subcutaneous WAT (sWAT) decreased 41% compared to control subjects who did not go through calorie restriction. A continued 2-week refeeding period dramatically increased sWAT ANGPTL8 level by 148%, suggesting that ANGPTL8 is induced upon refeeding and nutritionally regulated<sup>207</sup>.

Insulin is a strong inducer of ANGPTL8 expression in both AT and liver. ANGPTL8 transcript expression is dramatically induced by insulin during energy storage scenario, especially during TG formation and/or lipogenesis. An *in vitro* study has shown elevated ANGPTL8 mRNA expression after insulin induction along with glucose in cultured HepG2 cell<sup>208</sup>. Our previously reported insulin clamp study conducted on hyperinsulinemic human subject had recorded an induced expression of AT ANGPTL8 transcript. This specific effect was reconfirmed *in vitro* in SGBS adipocytes where insulin stimulated ANGPTL8 mRNA expression by 10-fold and protein expression by 32%<sup>34</sup>. Thyroid hormone had also been shown to upregulate ANGPTL8 expression in HepG2 cells<sup>209</sup>. Irisin, a small polypeptide myokine upregulates ANGPTL8 mRNA expression in 3T3-L1 adipocytes and intraperitoneal injection of recombinant irisin to mice also induced ANGPTL8 expression in AT<sup>210</sup>. siRNA-mediated knockdown of PPAR- $\gamma$  and SREBP-1c resulted in reduced expression of ANGPTL8 in 3T3-L1<sup>27</sup> and HepG2 cells<sup>211</sup> respectively, displaying a putative direct positive regulation on ANGPTL8. Moreover, tumor necrosis factor-alpha (TNF- $\alpha$ ),

a prominent proinflammatory cytokine, downregulates ANGPTL8 expression in 3T3L-1 adipocytes<sup>27</sup>.

#### *2.4.2.2. Secretion and intracellular localization*

ANGPTL8 bears a predicted N-terminal signal sequence from 1-20 amino acids suggesting that it is a secretory and/or membrane bound protein<sup>212</sup>. ANGPTL8 can be detected in serum in both human and mice and its levels in serum has been analysed in association with serum TG, VLDL, HDL and other metabolic markers, which is discussed in section (2.1.1.3). Cellular ANGPTL8 has been shown to colocalize along with lipid droplet and/or lipid droplet-linked cellular compartments<sup>209</sup>. ANGPTL8 localization has been reported at vesicular structures of varying shapes and sizes. In hepatoma cells, ANGPTL8 is distributed as small vesicle-like structures typically less than 1 $\mu$ m in diameter in the cytoplasm<sup>209</sup>. Larger ANGPTL8 vesicles are shown to localize with the lipid droplet protein perilipin 2 (PLIN2) or/and lysosome associated membrane protein 2 (LAMP2)<sup>209</sup>.

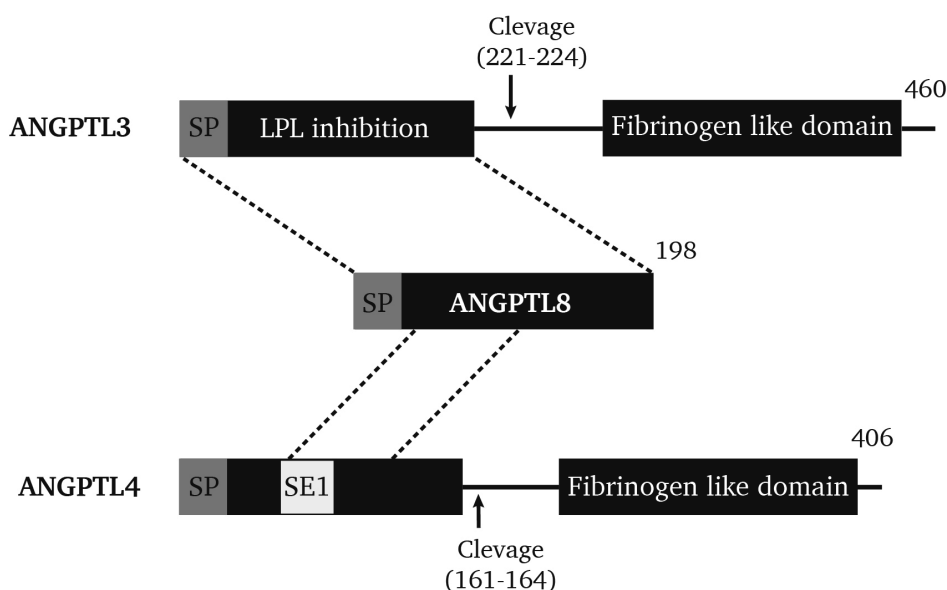
#### *2.4.3 Function of ANGPTL8*

Studies reporting functional characterization of ANGPTL8 started emerging during 2012. Three different research groups independently identified this protein and named as Lipasin, RIFL<sup>27,28</sup> and betatrophin. So far, ANGPTL8 has been studied by various groups as a major modulator of lipid metabolism along with the studies showing its role in insulin resistance<sup>208</sup>, autophagy<sup>209</sup> and regeneration of pancreatic beta cells (controversial). *In vitro* studies have revealed that serum TG levels were decreased when ANGPTL8 was knocked down, and overexpression of ANGPTL8 increased serum TG in mouse model studies<sup>27,29,200</sup>. Increased plasma TG levels in the presence of ANGPTL8 are explained by its ability to inhibit LPL leading to the slowing down of plasma TG clearance<sup>28</sup>. This concept was strengthened when ANGPTL8 was reported to decrease postprandial LPL activity in heart and skeletal muscles<sup>213</sup>. Inactivation of ANGPTL8 in mice through an antibody elevated the postprandial heparin LPL activity<sup>214</sup>. In addition to ANGPTL8's significant role in fed state, loss



of ANGPTL8 had been reported to distinctly reduce the absorption of VLDL-derived TG in AT upon refeeding.

Circulating levels of ANGPTL8 has been studied extensively in the context of human pathophysiology. Serum ANGPTL8 levels in humans were discovered to be low after overnight fasting<sup>29</sup> and elevated at 2 hours after refeeding<sup>32</sup>. Increased circulating ANGPTL8 levels were detected in T2D, child obesity with insulin resistance, T1D and gestational diabetes. However, due to the lack of detailed and consistent evidence, the connection between circulating ANGPTL8 and obesity and diabetes still needs to be established. Several ANGPTL8 sequence variations have been shown to be associated with lipid profiles by human genome wide association studies signifying that ANGPTL8 involved in both promoting and partitioning postprandial flux of TGs into AT<sup>213</sup>. ANGPTL8 in adipocytes plays a lipogenic function and promotes TG storage. ANGPTL8 expression positively correlates with adipogenesis in both human and mice and ANGPTL8 knockout mice showed reduced lipid storage in adipocytes during adipogenesis<sup>27</sup>. ANGPTL8 expression is higher in AT compared to muscle and heart. However, lack or inhibition by ANGPTL8 increases LPL activity in muscle and heart but not in WAT<sup>27,29,213,214</sup>. ANGPTL8 expression is induced by insulin plus glucose in 3T3-L1 adipocytes; however it was not detected as a secreted protein in the culture medium.<sup>27</sup> Interestingly, both feeding and cold exposure resulted in simultaneous rise in ANGPTL8 expression and the activity of LPL in WAT and BAT, respectively<sup>27,29,204,215,216</sup>. These data and more studies in parallel have revealed that ANGPTL8 is responsible for fine-tuning LPL activity and substrate partitioning by inhibiting LPL activity upon the fasting-feeding metabolic shift<sup>200,213</sup>. Apart from TG metabolism, ANGPTL8 had previously been suggested to stimulate pancreatic beta-cell proliferation. However, subsequent studies failed to reproduce the original finding; hence the concept of ANGPTL8 enhancing pancreatic beta-cell content has been invalidated<sup>217,218</sup>.



**Figure 4. ANGPTL8 homology with ANGPTL3 and ANGPTL4.** ANGPTL8 shares homology with N' terminal domain of ANGPTL3 which is crucial for serum TG regulation. ANGPTL8 also shares homology with N' terminal domain of ANGPTL4 which facilitates LPL binding. Spotted lines indicate homologous domains. LPL, lipoprotein lipase; SE1, a segment that mediates LPL binding. SP, signal peptide. This figure is adapted and modified from Zhang R, Biochem Biophys Res Commu. 2012 Aug 10;424(4):786-92

#### 2.4.4 The model of ANGPTL8, ANGPTL3 and ANGPTL4

TG trafficking is tightly regulated by LPL. During starvation, LPL activity is low in WAT and high in muscles. In contrast, in fed state, LPL activity increases in WAT and decreases in muscle. However, the mechanisms regulating the tissue specific function of LPL in fed/fast cycle remains to be addressed. The current scientific findings are depicting a combined working model where ANGPTL8, ANGPTL3 and ANGPTL4 are distributing the circulating TGs to appropriate organs under different physiological conditions<sup>214</sup>. ANGPTL8 activates ANGPTL3 to inhibit LPL activity in heart and skeletal muscle, while ANGPTL4 inhibits LPL activity in WAT. Fasting state elevates ANGPTL4 activity and downregulates ANGPTL8, and

subsequently LPL activity is reduced in WAT but increased in muscle. Hence, TGs are diverted towards muscle for oxidation<sup>29,213,219</sup>. On the other hand, refeeding reduces ANGPTL4 but upregulates ANGPTL8 activity. This increases LPL activity in WAT but reduces it in muscle, thus TGs are circulated to WAT for storage<sup>214</sup>

#### 2.4.5 ANGPTL8 in adipocytes and in metabolic diseases

In adipocytes, ANGPTL8 is strongly upregulated upon food intake and insulin stimulation, and downregulated by agents inducing lipolysis, like forskolin. A study has reported drastic upregulation of the ANGPTL8 transcript in AT of ob/ob mice relative to wild type mice<sup>27,28</sup>. ANGPTL8 is also expressed in mouse BAT and upregulated during cold exposure<sup>204</sup>. LPL activity is high in both of the above-mentioned conditions in WAT and BAT along with elevated expression of ANGPTL8. Hence one could argue that ANGPTL8 might possess LPL-independent functions in AT. Also, ANGPTL8 expression is strongly upregulated during the adipogenesis and its knockdown has been reported to hamper the process of adipocyte differentiation in 3T3-L1 cells<sup>27</sup>. Existing literature suggest that ANGPTL8 levels are upregulated in circulation during diabetes. Generally, diabetes is depicted by hypertriglyceridaemia, lower postprandial lipaemia and HDL cholesterol<sup>220</sup>. Given the ANGPTL8-3-4 model, one could reason that LPL inhibition by ANGPTL8 in heart and muscle resulting in hypertriglyceridemia could be the cause for diabetic dyslipidaemia. The question is thus whether diabetic dyslipidaemia could be treated by ablating ANGPTL8. Studies suggest that ANGPTL8 KO in mice do not have major health problems<sup>213</sup>; identification of homozygous null mutation in humans might reveal insights about the role of ANGPTL8 in human pathophysiology.

#### 2.4.6 ANGPTL8 - unanswered questions

Despite the recent major findings in the field of ANGPTL8 there are several crucial aspects to be addressed.

1. Intracellular function in adipocytes.
2. Mechanism of LPL inhibition by ANGPTL8.
3. Tissue specific inhibition of LPL according to the ANGPTL8-3-4 model<sup>221</sup>.
4. Identification of tissue specific transcription factors controlling ANGPTL8 expression and their binding sites.

### **3. Aims of the study**

1. To elucidate the functional role of miR192\* in adipocyte differentiation and TG storage.
2. To study the miRNA regulation of **ANGPTL8** expression in human subjects and Simpson Golabi Behmel Syndrome (SGBS) adipocytes.
3. To characterize the function of ANGPTL8 in 3T3L1 adipocytes.

## 4. Materials and methods

### 4.1 List of published methods

<i>Method</i>	<i>Studies used in</i>
RNA isolation	I, II, III
Cell culture	I, II, III
Stable overexpression of miR192* in SGBS cells	I
Triglyceride quantification	I, II
Microarray experiments and data analysis*	I
Bioinformatic analysis*	I, II
Quantitative real time reverse transcription PCR	I, II, III
Validation of microRNA targets	I, II
Antibodies and western blotting	I, II, III
Statistical analysis	I, II, III
Lipolysis assay	III
Lipidome analysis*	III
Staining of lipid droplets	III
Determination of plasma ANGPTL8 protein level*	II
Transfection experiments	III
Microscopy	III
Study subjects*	I, II
VAT biopsies*	I, II
Ethics	I, II

## 4.2 Brief descriptions of the methods employed

### 4.2.1 Cell culture

#### 4.2.1.1 SGBS (human) cell culture

SGBS preadipocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) F12 (Sigma-Aldrich, St. Louis, MO) at a 37 °C humidified incubator in 5% CO<sub>2</sub> and supplemented with 33 µM biotin, 17 µM pantothenate, 10% foetal bovine serum (Gibco/Life Technologies, Grand Island, NY), 100 I.U./ml penicillin, and 100 µg/ml streptomycin (Sigma Aldrich). Differentiation into mature adipocytes was carried out in a sequential manner with quick differentiation medium for four days followed by 3FC medium in serum-free conditions by employing a previously established 14-day protocol<sup>222,223</sup>.

#### 4.2.1.2 3T3-L1 (mouse) cell culture

3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-6429, Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Sigma-Aldrich) and 100 I.U./ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich). After 70% confluency cells were transduced with lentiviral particles expressing shRNA targeting Angptl8, Clone1: AAGCCCACCAAGAATTTGAGA, Clone2: TATGACAGAGCACTGGAATTC (CSTVRS-MissionTM, Sigma-Aldrich) and the transduced cell pools were selected using 3 mg/ml of puromycin. Following selection, 3T3-L1 cells were grown till confluency and differentiated to mature adipocytes using following steps: Cells were treated with 100 nM insulin, 500 µM 3-isobutyl-1-methyl- xanthine (IBMX), 250 nM dexamethasone, and 2 µM rosiglitazone in complete growth medium for the first 48 h, then replaced with 100 nM insulin and 2 µM rosiglitazone in complete growth medium for the next 48 h. Fresh complete growth medium was then changed every 48 h till day 6–8. Mature adipocyte cultures were quality checked for more than 80% differentiation from the abundant presence of large cytoplasmic lipid droplets then used for experiments.

#### *4.2.1.3 Human primary adipocyte culture*

Human subcutaneous preadipocytes obtained from a non-diabetic Caucasian male with BMI<30 and age<40 (Zen-Bio, Inc., Research Triangle Park, NC) were cultured with the preadipocyte medium (Zen-Bio, Inc.) in a 37°C humidified incubator with 5% CO<sub>2</sub>. After growing for 24 hrs, cells were checked for 100% confluency and differentiated into mature adipocytes according to manufacturer's instructions using differentiation medium (Zen-Bio, Inc.). Cells attained round morphology and presence of large lipid droplet formation was apparent after two weeks of differentiation. Mature adipocytes were incubated with renewed adipocytes medium (control) or new medium containing 2.5% macrophage conditioned media (MCM). In each treatment, new RPMI medium was diluted with adipocytes media to attain identical conditions. After 48 h of treatment, the cells were collected and stored at -80°C for future analysis.

#### *4.2.1.4 Human monocyte cell culture*

The human monocyte cell line THP-1 (ATCC) was cultured in RPMI 1640 medium with 10% fetal bovine serum, 2 mM glutamine, 5 mM glucose, 50 mg/ml Gentamycin and 20 mM HEPES, pH 7.4, at humidified 37°C in a 5% CO<sub>2</sub> as previously described<sup>224</sup>. Type 1 macrophage-like state (M1) was induced with 0.162 mM phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO) for 24 hrs. Differentiated cells were washed with cold Dulbecco's phosphate-buffered saline and incubated with new medium without PMA. Then, differentiated M1 macrophages were treated with fresh medium containing 10 ng/ml lipopolysaccharide (LPS, Sigma Chemical Co.) for 24 hrs. The LPS-stimulated macrophage conditioned media (MCM) was then collected and centrifuged at 400 x g for 5 min, (Zen-Bio, Inc.), diluted with adipocyte medium and used to induce inflammation in mature human adipocytes as described<sup>224</sup>. HuH7 cells were cultured at a 37 °C humidified incubator in 5% CO<sub>2</sub>, in AQMEM medium (Sigma-Aldrich), 10% fetal bovine serum (Sigma-Aldrich) 100 I.U./ml penicillin, and 100 µg/ml streptomycin (Sigma Aldrich).



#### *4.2.1.5 Lentiviral transduction*

miR-192\* lentiviral constructs shMIMIC® (VHS5841-101207549), or a nontargeting miRNA controls (HRM5872, Thermo Scientific) were transduced into SGBS fibroblasts.

Lentiviral particles at a multiplicity of three I.U./cell were infected in serum free condition with DMEM mentioned above with 8.0 µg/ml hexa dimethrine bromide (Sigma Aldrich). Growth medium was added on top after 4 h of incubation. The cells were allowed to grow for 48 hrs, then subjected to selection by adding growth medium containing 0.5 µg/ml puromycin (Sigma-Aldrich). The cells were expanded in the presence of puromycin and then subjected to adipogenic differentiation, during which no antibiotic selection was applied.

#### *4.2.1.6 Transfection*

SGBS preadipocytes were transfected with miRNA mimics or a non-targeting control siRNA (see above) in 6-wells for 24 hrs using the RNAiMax™ reagent (Thermo Fisher Scientific, Waltham, MA). Adipogenic differentiation was then induced according to a published protocol<sup>222</sup> without replacing the transfection complexes. Adipocyte cultures were harvested for western analysis after 4 days.

#### *4.2.1.6 Lipolysis assay*

Angptl8 knockdown and control 3T3-L1 cells were incubated under basal and isoproterenol (10 µM) treated conditions for 2 hrs, after which the cell supernatants were measured for the release of non-esterified fatty acids (NEFA) using HR Series NEFA-HR kit (Wako Diagnostics, Richmond, VA) according to the manufacturer's protocol.

#### 4.2.2 Gene expression analysis

##### 4.2.2.1 Total RNA isolation form AT

Frozen AT biopsies (~200 mg) were thawed on ice to 4°C and homogenized in 0.6 ml QIAzol® lysis solution QIAGEN (Germantown, MD). Total RNA was extracted and purified using miRNeasy® Mini Kit QIAGEN (Germantown, MD). Final RNA concentrations were estimated with a NanoVue (GE Healthcare Life Sciences Pittsburgh, USA)

##### 4.2.2.2 Total RNA isolation form cell culture lysates

Cells were lysed directly from culture plates by using RNA lysis buffer and vortexed briefly to ensure lysis. Total RNA was isolated using PureLink® RNA Mini (Ambion/Thermo Fisher, Waltham, MA) or RNeasy® Mini Kit QIAGEN (Germantown, MD). Final RNA concentrations were estimated NanoVue (GE Healthcare Life Sciences Pittsburgh, USA)

##### 4.2.2.3 Microarray data analysis

Background noise correction within arrays was done using a normal exponential convolution model<sup>225</sup>. Array data were normalized by quantile process to confirm that intensity values have the same empirical distribution through arrays. Box plot and density plot of array data were used for quality control. Data were log2 transformed. Differential expression between SGBS-miR192\* and SGBS-NT adipocytes was equated by empirical Bayes statistics<sup>226</sup>. P-values were corrected for multiple testing by the Benjamini–Hochberg method. The data is available at <http://www.ncbi.nlm.nih.gov/geo/> with access code GSE62951.

##### 4.2.2.4 qRT-PCR

Total RNAs isolated from cell cultures were reverse transcribed using Exiqon Universal® or VILO® reverse transcriptase (Invitrogen, Carlsbad,

CA) kits. Real time qPCR was performed in LightCycler®480 (Roche Applied Science, Penzberg, Germany) using specific miRNA primer (Exiqon), gene-specific primer pairs (Sigma Aldrich) and the Roche SYBRGreen® master mix. As housekeeping references, 36B4 (acidic ribosomal phosphoprotein) and SDHA (succinate dehydrogenase complex, subunit A) genes were used. The crossing points (Cp) values were evaluated for every amplification curve using Second Derivative Maximum Method<sup>227</sup>.  $\Delta C_p$  value was initially calculated by subtracting the Cp value for equivalent endogenous controls in each sample from the Cp value for each sample and target gene. Fold changes compared with the endogenous control were then determined by calculating  $2^{-\Delta C_p}$ . Duplicates and positive and negative controls were included.

#### 4.2.2.5 TaqMan qRT-PCR

Isolated RNA samples were reverse transcribed using TaqMan® microRNA Reverse Transcription Kit (Life Technologies/Applied Bioscience, Foster City, CA) or VILO® reverse transcriptase (Invitrogen, Carlsbad, CA) kits. miRNA expression was analysed with miRNA specific TaqMan® MicroRNA assays by using RNU44 as a reference gene. Gene expression analysis was performed using commercially available and pre-validated TaqMan® primer/probe sets: RNU44 (assay# 001094) and ribosomal protein lateral stalk subunit P0 (RPLP0, Hs99999902\_m1) were used as endogenous controls for miRNA and gene expressions, respectively.

### 4.2.3 Protein detection and quantification

#### 4.2.3.1 Western blot

Cultured cells were lysed in radioimmunoprecipitation assay buffer (RIPA) with protease inhibitor cocktail, sonicated, and centrifuged to remove the insoluble material. Protein concentrations were subsequently measured from the supernatant. Protein samples were then boiled for 5 min along with reducing Laemmli sample buffer. Proteins were subjected to electrophoresis on SDS poly- acrylamide gels and electrotransferred on

Hybond-C Extra nitrocellulose membrane. Samples on nitrocellulose membrane were treated with primary antibodies followed by horseradish peroxidase conjugated secondary antibodies to visualize the proteins of interest using enhanced chemi-luminescence on Fujifilm X-ray films. The detected bands were then digitally scanned and analysed using Image J software<sup>228</sup>.

#### *4.2.3.2 ELISA*

The quantification of ANGPTL8 in heparin plasma was determined by using a commercial ELISA assay (Aviscera Bioscience, Santa Clara, CA)

#### *4.2.4 Lipid analysis*

##### *4.2.4.1 Triglyceride quantification assay*

SGBS adipocytes were lysed using RIPA buffer with protease inhibitor cocktail and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was then subjected to triglyceride analysis using the GPO-PAP Triglyceride assay kit (Cobas, Roche/Hitachi, Tokyo, Japan). The data was normalized to the total protein concentration.

##### *4.2.4.2 Lipid droplet staining.*

Mature adipocytes were washed and fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100 in phosphate-buffered saline. Cells were then stained with 100 nM Bodipy 493/503 (Molecular Probes/Life Technologies, Eugene, OR) for one hour. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were imaged using Zeiss Axio Observer Z1 microscope (Carl Zeiss Imaging Solutions GmbH, Oberkochen, Germany).

#### 4.2.4.3 Lipidome analysis

Lipids were subjected to direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode using the analytical setup and strategy described in<sup>229</sup>. A precursor ion of  $m/z$  184 was employed for phosphatidylcholine (PC), sphingomyelin (SM)<sup>229</sup> and lysophosphatidylcholine (LPC)<sup>230</sup>. The following neutral losses were applied: PE 141, phosphatidylserine (PS) 185, phosphatidylglycerol (PG) 189 and phosphatidylinositol (PI) 277<sup>231,232</sup>. PE-based plasmalogens (PE P) were quantified based on the principles explained in<sup>233</sup>. Lipid species were interpreted according to the newly published proposal for shorthand notation of lipid structures that are derived from mass spectrometry<sup>234</sup>. Glycerophospholipid species annotation was based on the hypothesis of even numbered carbon chains.

#### 4.2.5 Molecular biology

##### 4.2.5.1 Cloning of luciferase constructs

SCD and ALDH3A2 3'UTR constructs were acquired from GeneCopoeia (Rockville, MD). The miR-192\* target sequences SCD and ALDH3A2 were mutagenized using QuikChange XL kit (Agilent, Santa Clara, CA). The ANGPTL8 3'UTR DNA was PCR amplified and cloned downstream of firefly luciferase in the dual luciferase vector pEZX-MT06 (GeneCopoeia, Rockville, MD). Mutagenesis to remove the predicted miR-221-3p binding site was performed by using the Quick Change XL kit (Agilent, Santa Clara, CA).

##### 4.2.5.2 Dual luciferase assay

HuH7 cells in 12-wells were transfected with the Luc-3'UTR constructs along with miRNA mimics (100 nM) or a non-targeting control siRNA (100 nM) by using Lipofectamine 2000<sup>TM</sup> (Invitrogen, Carlsbad, CA) for 24 hrs. Cells were lysed in passive lysis buffer (from the luciferase kit) and firefly and Renilla luciferase activities were measured by using the

Dual Luciferase kit (Promega, Madison, WI). The firefly values were normalized by using the Renilla values.

### 4.2.6 Subject recruitment

#### 4.2.6.1 Cohort 1:(I)

Morbidly obese subjects (BMI  $46.8 \pm 6.3$ ) were recruited from the group of patients undergoing laparoscopic bariatric surgery in Peijas Hospital of the Hospital District of Helsinki and Uusimaa. The following criteria were considered during the process of recruitment: (i) age 18–65 years; (ii) no reported acute or chronic disease excluding obesity or obesity related diseases such as T2D, non-alcoholic fatty liver disease, CVD and/or hyperlipidaemia; Elimination criteria were: (i) alcohol abuse ( $>20$  g/day), (ii) hepatotoxic medications or herbal products usage, and (iii) lactation or pregnancy. The subjects were examined in the morning after an overnight (10–12 h) fast one to two weeks before the surgery. Height, weight, hip and waist circumferences were documented as described<sup>235</sup>. Blood samples were drawn to measure the concentrations of (HDL) and (LDL) cholesterol, TG, glucose, C-peptide, and insulin, along with alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) activities as described<sup>236</sup>. Prior to operation, patients adhered to a 6–12 week of very low-calorie diet (800 kcal/day). Detailed clinical characteristics of the subjects are shown in Table 2.

#### 4.2.6.2 Cohort 2: (I, II)

Subjects were (BMI  $31.2 \pm 2.5$ ) 17 female cohort, undergoing elective gynecological surgery for non-malignant causes in the department of Obstetrics and Gynecology, Helsinki University Hospital, were recruited. Patients were scheduled for abdominal or laparoscopic hysterectomy, diagnostic laparoscopy, salpingo-oophorectomy, enucleation of uterine myoma, or laparoscopic colpo-sacro-rectopexy or enucleation of ovarian cyst. Causes for surgery comprised uterine myomas accompanied with menorrhagia and/or pain, benign ovarian cysts, infertility and

dysmenorrhea, or rectocele. Blood samples were collected before the surgery and centrifuged to isolate serum and stored at  $-20^{\circ}\text{C}$ . Detailed clinical characteristics of the subjects are shown in Table 3.

#### *4.2.6.3 Cohort 3 (II):*

Subjects were recruited at the department of Diabetes, Endocrinology and Nutrition and the Department of Surgery of the Hospital “Dr Josep Trueta” of Girona (Girona, Spain) Abdominal SAT samples were collected from 69 women [Body mass index (BMI) =  $39.6 \pm 10.9 \text{ kg/m}^2$ , age =  $49 \pm 12$  years (mean  $\pm$ SD) during elective surgery (cholecystectomy and surgery of abdominal hernia) at the Hospital “Dr Josep Trueta” of Girona (Table 4). SAT biopsies were also withdrawn from 22 morbidly obese women before (‘baseline-longitudinal’ or ‘pre-bariatric surgery’) and ca. two years after surgery-induced weight loss were analysed. (Roux-en-Y gastric bypass; Table 4). MicroRNA and gene expressions were measured (qRT-PCR) on all AT biopsies as previously explained<sup>237</sup>. Recruits were previously informed and written consent was signed. Subjects were of Caucasian origin and their reported body weight was stable for at least three months before the study. No systemic diseases were reported other than obesity and/or T2D. All participants were devoid of any infections one month before entering the study. Liver and thyroid dysfunction were specifically excluded. Other exclusion criteria were: i) history of drug or alcohol abuse, or serum transaminase activity more than twice the upper limit of normal, ii) clinically significant hepatic, neurological, or other major systemic disease, including malignancy, iii) age above 65 or below 25 years, iv) acute major cardiovascular event in the previous 6 months, v) an elevated serum creatinine concentration, vi) acute illnesses and current evidence of high grade chronic inflammatory or infective diseases, and vii) mental illness rendering the subjects unable to understand the nature, scope, and possible consequences of this study. The study protocol was approved by the Ethics Committee and the Committee for Clinical investigation (CEIC) of the Hospital “Dr. Josep Trueta” of Girona.

#### 4.2.7 Bioinformatics and statistics

Kolmogorov–Smirnov test was used to examine the clinical readouts and normality of distribution. Non-normally distributed data are mentioned as median (25%–75% percentile) normally distributed data are represented as means  $\pm$  standard error of mean (SEM). Spearman's test was used to analyse the correlations between miR-192\* and miR-192 expression levels in VAT. miRNA binding region in the 3'UTR of ANGPTL8 mRNA (NM\_018687.6) were located by using Targetscan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/), Whitehead Institute for Biomedical Research, Cambridge, MA). Correlation analysis of miRNA and gene were analysed by nonparametric Spearman test in Prism 7 (GraphPAD, La Jolla, CA) software. Gene expression between human study subjects were analysed using Mann–Whitney test in Prism 7 (GraphPAD, La Jolla, CA) and Ingenuity™ pathway analysis software. Results of continuous variables are stated as mean $\pm$ standard deviation; p-values <0.05 were mentioned as statistically significant.

Table 2

Anthropometric and biochemical data of Cohort 1

##### **Bariatric surgery Cohort**

Gender (females/males)	18/9
Age (years)	48.0 $\pm$ 9.7
Body weight (kg)	133.5 $\pm$ 25.9
Body mass index (kg/m <sup>2</sup> )	46.8 $\pm$ 6.3
<b>Biochemical parameters</b>	
fP-glucose (mmol/L)	5.8 $\pm$ 0.9
fS-insulin (mU/L)	11.9 (7.3–20.0)
fS-C-peptide (nmol/L)	1.1 $\pm$ 0.40
fS-LDL cholesterol (mmol/L)	2.8 $\pm$ 0.9
fS-HDL cholesterol (mmol/L)	1.2 $\pm$ 0.3
fS-TG (mmol/L)	1.3 $\pm$ 0.5
fS-ALT (U/L)	34 (22.0–51.5)
fS-AST (U/L)	30 (24.0–39.5)



fS-ALP (U/L)	69 (56.0–82.5)
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Table 3

Anthropometric and biochemical data of Cohort 2

<b>Gynecologic surgery cohort</b>	
Gender (females/males)	17/0
Age (years)	46.0 ± 9.5
Body composition	
Body weight (kg)	85.0 ± 9.0
Body mass index (kg/m <sup>2</sup> )	31.2 ± 2.5
Biochemical parameters	
fS-LDL cholesterol (mmol/L)	3.4 ± 0.8
fS-HDL cholesterol (mmol/L)	1.4 ± 0.4
fS-TG (mmol/L)	1.6 ± 2.0
fS-AST (U/L)	33 (21–145)

Table 4

Anthropometric and biochemical data of Cohort 3

<b>Parameters</b>	
N	69
Age (years)	49 ± 12*
BMI (kg/m <sup>2</sup> )	39.6 ± 10.9
Fat mass (%)	53.4 ± 12.4
Glucose (mg/dl)	103.4± 35.7**
HbA1c (%)	5.3 ± 2.1
Cholesterol (mg/dl)	197.7 ± 36.8
LDL (mg/dl)	117.7 ± 29.3
HDL (mg/dl)	57.1 ± 15.0
Triglycerides (mg/dl)	113 ± 43.4

<b>Parameters (BMI&lt;30)</b>	
N	21
Age (years)	56 ± 13
BMI (kg/m <sup>2</sup> )	26.3 ± 2.6
Fat mass (%)	40 ± 4.5
Glucose (mg/dl)	99.3 ± 26.0
HbA1c (%)	5.4 ± 0.6
Cholesterol (mg/dl)	222.2 ± 40
LDL (mg/dl)	132.3 ± 25.5
HDL (mg/dl)	62.3 ± 18.0
Triglycerides (mg/dl)	106 ± 38.7

<b>Parameter (BMI&gt;30)</b>	
N	48
Age (years)	46 ± 10
BMI (kg/m <sup>2</sup> )	45 ± 7.4
Fat mass (%)	59.7 ± 9
Glucose (mg/dl)	105 ± 39.2
HbA1c (%)	5.1 ± 2.6
Cholesterol (mg/dl)	187.8 ± 33.1
LDL (mg/dl)	111.2 ± 28.8
HDL (mg/dl)	54.9 ± 13.2
Triglycerides (mg/dl)	115 ± 42.5

Abbreviations: ALT alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, Body mass index; f, fasting; HDL, highdensity lipoprotein; LDL low-density lipoprotein; P, plasma; S, serum; TG, triglycerides; Data are shown as mean ± SD or median (25%–75% percentile), as applicable. \*The values represent mean ± SD. BMI, body mass index; HbA1c, glycated hemoglobin; HDL, highdensity lipoprotein; LDL, low-density lipoprotein \*\* Of the subjects, 9 were on metformin and 2 on metformin plus insulin therapy.

## 5. Results

### 5.1 Study I

The primary objective of Study I was to investigate the molecular regulators of AT lipid metabolism, focusing on the function of miR-192\* in human adipocyte lipid metabolism.

#### 5.1.1 Association between miR-192\* expression and adipose tissue in human cohorts.

Our miRNA profiling study from VAT of (cohort I) obese subjects undergoing bariatric surgery exhibited a negative correlation between VAT miR-192\* expression and serum TG levels (Fig. 1A, I) and a positive correlation with serum HDL cholesterol (Fig. 1B, I) and aspartate amino transferase (Table 3, I). miR-192 showed similar correlation trends with TG and HDL; however, these were not significant. Positive correlation between miR-192\* and miR192 suggested similar abundancy of both mature miRNAs originating from same pre-miRNA. Insertion of covariates such as BMI, age and gender did not significantly alter the correlation between miR-192\* with HDL/TG (data not shown). No correlation was found between miR192\* and BMI among morbidly obese subjects. In correlation analysis from VAT biopsies of Cohort 2, the lower BMI group ( $31.2 \pm 2.5$ ) showed no significance correlation between miR-192\* with serum HDL, TG or AST (Table 4, I); however, this group displayed a significant negative correlation between VAT miR-192\* and BMI (Fig. 1D, I). Relatively low expression of miR-192\* was observed compared to miR-192 in subgroup of BMI >30 in cohort 2. Target prediction analysis suggested numerous genes involved in lipid metabolism which could be targeted by miR192\*, including peroxisome proliferator activated receptor  $\alpha$  (PPAR- $\alpha$ ), low-density lipoprotein receptor (LDLR), patatin-like phospholipase domain containing 3 (PNPLA3), Krüppel-like factor 6 (KLF6), caveolins 1 and 2 (CAV1, CAV2), stearyl coenzyme A desaturase

1 (SCD) and aldehyde dehydrogenase 3 family member A2 (ALDH3A2). With the existing data, we further continued to characterise the function of the 3' arm species, miR-192\*, in adipocytes

### 5.1.2 Functional characterization of miR192\* in SGBS adipocytes

Stable overexpression of miR-192\* (SGBS-miR-192\*) or non-targeting control (SGBS-NT) were established in SGBS pre-adipocytes using a lentiviral vector. Cells were then subjected to adipogenic differentiation. Successful overexpression of miR-192\* was confirmed by qPCR analysis in both pre-adipocytes and mature adipocytes (Fig. 2A, I). SGBS adipocytes expressing miR-192\* showed less TG storage (25%) than control adipocytes (Fig. 2B, I). This phenotype was further supported by the altered expression of key adipogenic markers. qPCR analysis revealed a marked reduction of GLUT4 mRNA and a negative trend for the AP2 (statistically not significant) (Fig. 2C, D, I). However, western blot analysis showed a significant reduction of AP2 and perilipin 1 (PLIN1) protein levels. These results clearly suggest that miR-192\* expression in SGBS adipocytes caused a defect in adipogenic differentiation and TG storage. We performed transcriptome profiling of SGBS adipocytes expressing miR192\* and NT control (n=4 each) to identify the pathways and specific target genes through which miR-192\* is causing the defect in adipocyte differentiation and TG storage. We found 637 mRNAs to be differentially expressed between SGBS-miR192\* and SGBS-NT out of which 176 mRNAs were downregulated in SGBS- miR-192\*. Pathway analysis was carried out on differentially expressed genes using the Ingenuity™ pathway analysis. Over- expression of miR192\* affected the 'LPS/IL-1 mediated inhibition of RXR function' and 'LXR/RXR activation' pathways (Fig. 3, I). Within these pathways, expression of 14 mRNAs were considerably dampened, and 13 mRNAs were upregulated in SGBS-miR192\*. Several key lipogenic genes were among the mRNAs which were reduced in SGBS-miR-192\*, including SREBF1 (sterol regulatory element binding protein-1), ACSL3 (acyl-coenzyme A synthetase long chain-3), ACSL6, SCD (stearoyl coenzyme A desaturase-1), FABP7 (fatty acyl binding protein-7). Several genes from the downregulated pathways

were predicted at least by two algorithms to be direct targets of miR-192\*. (Fig. 3, I). In addition to mRNA suppression caused by expression of miR-192\*, the expression of several mRNAs was elevated in the affected pathways, like IL1R2 (interleukin-1 receptor type II), APOH (apolipoprotein H), CYP3A5 (cytochrome P450 3A5), C9 (complement component 9) and MSR1 (macrophage scavenger receptor 1/scavenger receptor A).

### 5.1.3 Validation of targets of miR-192\*

In the last section, we performed validation experiments to identify and confirm miR-192\* targets from SGBS-miR-192\* and SGBS-NT adipocytes. We quantified both mRNA and protein expressions of relevant targets through a series of qPCR and western blot experiments, qPCR analysis confirmed the downregulation of FABP7, ALDH3A2, SCARB1, ALDH3B2, SCD and increased expression of APOH, CYP3A5, and MRS1 mRNAs in the SGBS-miR-192\* adipocytes (Fig. 4, I). Protein expression of SCD1, ALDH3A2, SCARB1 (SR-B1) were significantly reduced in SGBS-miR-192\* compared to the SGBS-NT adipocytes (Fig. 5, I). To confirm whether miR-192\* physically binds the SCD and ALDH3A2 mRNAs, we performed dual luciferase reporter assays. First we cloned the 3' UTRs (miRNA-192\* binding) of SCD and ALDH3A2 to luciferase reporter construct. miR-192\* mimics or non-targeting RNAs were co- transfected with luciferase constructs into HuH7 cells followed by optical measurement in luminometer. Both SCD and ALDH3A2 reporter constructs showed a marked reduction in firefly luciferase activity due to direct binding of miR-192\* on the respective 3'UTRs. We then introduced mutations to check the specificity of miR-192\* binding on these UTRs. The mutations disrupted the effects of miR-192\* on both SCD and ALDH3A2 3'UTR reporters, which confirms that SCD and ALDH3A2 are directly regulated by miR-192\*.

## 5.2 Study II

This study is focused on regulation of ANGPTL8 by miR221-3p under inflammation induced condition in adipocytes and influence of miR-221-3p expression in the context of dysregulated adipose tissue metabolism

### 5.2.1 ANGPTL8 in context with insulin sensitive and lipogenic markers.

In the light of several studies showing the insulin responsiveness of ANGPTL8, we analysed correlations of ANGPTL8 gene expression with hallmark insulin sensitivity markers like GLUT4 and ADIPOQ. We found a significant positive correlation with GLUT4 ( $r = 0.48$  and  $P < 0.0001$ ) in female SAT ( $n=69$ ) of cohort 2 (Fig. 1A, II), and in VAT ( $r = 0.63$ ,  $P = 0.004$ ) of cohort 1 (Fig. 1C II). In addition, ANGPTL8 positively correlated with ADIPOQ in SAT ( $r = 0.43$ ,  $P = 0.0003$ ) and VAT ( $r = 0.49$ ,  $P = 0.03$ ) (Fig. 1B and 1D II). We also found significant correlations in a subgroup analysis where BMI  $>30$ , and  $<30$  subgroups of cohort 2 were assessed distinctly (data not shown). In support to previously published data, ANGPTL8 also revealed a significant positive correlation with FASN and DGAT1 mRNAs, which are important lipogenic genes. (supplemental Fig. 1A and 1B II).

### 5.2.2 Inflammation, a key modulating factor of ANGPTL8 and miR-221-3p expression in human adipocytes

It is well known that inflammation is associated with the expression of genes stimulated by insulin and it is frequently related to IR status<sup>238,239</sup>. We investigated the possible influence of pro-inflammatory stimuli on ANGPTL8 expression in adipocytes. We created a physiologically relevant condition where we exposed human primary adipocyte culture to conditioned medium of LPS-stimulated THP-1 macrophages (MCM,

2.5%) or the corresponding control medium and analysed ANGPTL8 mRNA expression by qPCR. The pro-inflammatory treatment drastically reduced ANGPTL8 expression (by -86%,  $p < 0.001$ ) (Fig. 2A, *II*). We also validated the suppression of insulin responsive genes Adiponectin (-51%,  $p < 0.01$ ) and GLUT4 (-61%,  $p < 0.001$ ) upon treatment with 2.5% MCM (Fig. 2A, *II*). Upregulation of inflammatory facilitators interleukin 6 and tumour necrosis factor- $\alpha$  was observed, which increased 13- and 3-fold ( $p < 0.01$  and  $p < 0.001$ ), respectively (Fig. 2B). Interestingly, treatment of 2.5% MCM also suppressed mRNA levels of the key lipogenic genes, FASN and DGAT1.

miRNAs modulate the expression of several genes under inflammatory conditions<sup>240-243</sup>. Our hypothesis was to investigate whether miRNAs are regulating ANGPTL8 expression under such conditions. Hence we mapped the 3'UTR of ANGPTL8 for putative miRNA binding sites. The short 280-nt 3'UTR of ANGPTL8 revealed several miRNA binding sites which have previously been linked with insulin resistance, T2D or obesity: miR-107, miR-143-3p, and miR-221-3p (Fig. 2C, *II*). We found that only miR-221-3p expression was elevated (2.5-fold,  $p < 0.001$ ) in primary adipocytes under inflammatory conditions induced by 2.5% MCM treatment (Fig. 2D, *II*).

### 5.2.3 miR221-3p physically binds ANGPTL8 3'UTR and regulates its expression in adipocytes

We conducted dual luciferase assays to test the direct binding of miR-221-3p on the ANGPTL8 3'UTR which was previously cloned in Luc reporter constructs. Both miR-221-3p and NT mimics along with Luc constructs were transfected to HuH7 cells. After 24hr incubation, cells were lysed and measured for Luc signal. We observed a significant decrease (28.5%,  $p < 0.001$ ) in Luc signal in cells transfected with miR-221-3p compared to NT controls (Fig. 3A, *II*), which implies the direct binding of miR-221-3p on the ANGPTL8 3'UTR. We also confirmed that a mutation introduced into the miR-221-3p binding site successfully destroyed the binding capability, indicating the direct binding of miR-

221-3p to the ANGPTL8 3'UTR (Fig. 3B, *II*). To further confirm the action of miR-221-3p on ANGPTL8 in adipocytes, we transfected miR-221-3p mimics and non-targeting siRNA into SGSB preadipocytes and ANGPTL8 protein levels were measured after 4 days of differentiation. Western blotting revealed that the ANGPTL8 protein was dampened by 46% ( $p < 0.01$ ) in adipocytes transfected with miR-221-3p compared to non-targeting control (Fig. 3C and D, *II*). Interestingly, miR-221-3p transfection into SGSB cells also significantly reduced TG accumulation (Fig. 1C).

### 5.2.4 Condition-specific association between miR-221-3p and ANGPTL8 in AT in vivo

To investigate the possible relation between miR-221-3p with ANGPTL8 we analysed the expression of both miR-221-3p and ANGPTL8 in two human cohorts. SAT biopsies of cohort 2 consisting of female subjects ( $n=69$ ) (Supplemental Table 1, *II*) showed a marginal tendency of negative correlation between miR-221-3p with ANGPTL8 ( $r = -0.16$ ,  $p = 0.18$ ; Fig. 4A *II*). VAT biopsies from cohort 1, consisting of 19 women ( $BMI\ 31.6 \pm 3.2$ ) showed a similar tendency ( $r = -0.25$ ,  $p = 0.3$ ) (Supplemental Table 2; Fig. 4B *II*). Concurrently, subgroup analysis of the cohort 2 obese subjects ( $BMI > 30$ ,  $N = 48$ ) revealed a similar correlation trend ( $r = -0.19$ ,  $p = 0.18$ ; Fig. 4C *III*), which was not seen in the overweight subcategory ( $BMI < 30$ ,  $N = 21$ ,  $r = -0.03$ ; Fig. 4D *II*). We subsequently evaluated the mRNA and miRNA expression in a morbidly obese subcategory of cohort 2 ( $n=22$ ) that had undergone bariatric surgery. SAT biopsies were obtained from these subjects prior to surgery and after two years of post-operation weight loss follow-up (Table 1, *II*). Substantial weight loss following operation, ( $BMI$  from  $43.4 \pm 5.0$  to  $29.2 \pm 5.7$ ,  $p < 0.0001$ ), both serum lipid (HDL and TG) values and GLUT4 expression were significantly improved. Likewise, ANGPTL8 expression showed an increasing trend and miR221-3p expression was dampened but the difference was not statistically significant (Table 1, *II*).



However, an interesting observation was that a significant negative correlation was found ( $r = -0.51$ ,  $p = 0.016$ ) between miR221-3p and ANGPTL8 in biopsies collected from the morbidly obese subjects before bariatric surgery (Fig. 4E, II). This correlation was not observed in post weight loss AT biopsies (Fig. 4F, II). This bariatric surgery cohort was further subdivided considering with ( $n=7$ ) or without ( $n= 15$ ) metformin or metformin plus insulin therapy, which revealed a negative correlation between miR-221-3p and ANGPTL8 in the non-medicated group. Exclusion of the seven medicated patients further strengthened the negative correlation of miR-221-3p and ANGPTL8 mRNA ( $r = -0.70$ ,  $p = 0.003$ ), suggesting that the improved metabolic status and weight loss post bariatric surgery may influence the regulatory mechanisms of ANGPTL8 expression in AT.

#### 5.2.5 Association between ANGPTL8 and miR221-3p with AT inflammation *in vivo*

In a genome wide transcriptome study, Soronen et al. had previously identified that phospholipase A2 G7 (PLA2G7), an inflammatory marker, was strongly upregulated in obese insulin resistant subjects<sup>33</sup> and was also reported in other studies to be induced upon AT inflammation<sup>244-246</sup>. Hence, we quantified PLA2G7 mRNA expression in SAT biopsies of Cohort 2. Interestingly, PLA2G7 showed a significant negative ( $r = -0.50$ ,  $p = 0.01$ ) correlation with ANGPTL8 and positive ( $r = 0.57$ ,  $p = 0.006$ ) correlation with miR-221-3p (Fig 5A and 5b, II). This further supported our hypothesis that inflammation may control the miR-221-3p regulation of ANGPTL8 expression in AT. When analysed separately, considering PLA2G7 expression, we found a significant difference in ANGPTL8 mRNA quantity ( $p = 0.009$ ) and an almost significant trend in miR-221-3p ( $p = 0.06$ ) between the PLA2G7 low- and high-expression groups as divided at the median (Fig. 5C, II).

### 5.2.6 No direct relation between SAT and circulating ANGPTL8 levels

Increased ANGPTL8 levels had been associated with obesity related insulin resistance and diabetes. Hence, we quantified the ANGPTL8 protein levels in heparin plasma from a bariatric surgery patient group ( $n = 16$ ). We found no correlation between the SAT ANGPTL8 mRNA and the plasma ANGPTL8 concentration at baseline ( $r = -0.13$ ,  $p = 0.96$ ) nor after weight loss ( $r = 0.020$ ,  $p = 0.93$ ). As an interesting observation, plasma ANGPTL8 levels elevated significantly after the weight loss (baseline  $105.6 \pm 19.9$  ng/ml, after weight loss  $119.8 \pm 17.6$  ng/mL;  $P = 0.049$ ).

## 5.3 Study III

This study aims investigate the intracellular function of Angptl8 in 3T3L1 adipocytes.

### 5.3.1 Angptl8 knockdown in 3T3-L1 adipocytes alters lipid storage and composition

To investigate the function of ANGPTL8 in adipocyte lipid metabolism, we established two stable ANGPTL8 knockdown cell pools using independent lentiviral shRNA constructs termed as Angptl8 KD1 and Angptl8 KD2. ANGPTL8 knockdown cells were carefully grown under selection pressure and confirmed for the knockdown efficiency (approximately 70-75%) for both KD1 and KD2 by measuring mRNA expression by qPCR (Fig. 1A, *III*). We quantified TGs in the ANGPTL8 knockdown cells using thin layer chromatography and documented a 18-19% reduction compared to cells transduced with non-targeting shRNA control lentivirus (Fig. 1B, *III*). We checked the mRNA expression of genes encoding TG synthesizing enzymes monoacylglycerol O-Acyltransferase-1 MOGAT1, diacyl-glycerol O-Acyltransferase-1 (DGAT1),

glycerol-3-phosphate acyltransferase-1 (GPAT1) and found no reduction. Lipid droplet (LD) staining with Bodipy 493/503 (green) dye showed a similar general LD morphology in both Angptl8 KD1, KD2 and control cells (Fig. 1C, E, *III*). We observed a subtle difference in KD1 cells where cell size and LD distribution were heterogeneous with many small sized LDs in some cells and few large LDs in another cell population (Fig. 1D, *III*).

To study the comprehensive effect on ANGPTL8 knockdown, we obtained total lipid from both ANGPTL8 KD1, KD2 and control cells and performed quantitative direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis. The results obtained were analyzed independently in KD1 and KD2 with shRNA controls. We found disparities in lipid species among KD1 and KD2 and also found substantial differences between ANGPTL8 KD1, KD2 and the shRNA control. We found a decline of ether phospholipids (alkyl-PCs and PE plasmalogens) in the KD cell pools (Fig. 2A–E, *III*). In addition, some very low abundant lipid species like saturated PCs, lysoPCs and PEs, were significantly reduced in both Angptl8 KD pools (Fig. 2F–H, *III*).

### 5.3.2 Angptl8 knockdown alters lipolysis and lipid oxidation in 3T3-L1 adipocytes

It is well known that Angptl8 inhibits LPL in circulation together with Angptl3<sup>29</sup>. Along with our results which showed reduced TG accumulation in Angptl8 knockdown, this encouraged us to analyse the intracellular lipolysis in Angptl8 knockdown and control 3T3-L1 adipocytes when stimulated with isoproterenol ( $\beta$ -adrenergic receptor agonist). We measured the release of NEFA in the culture medium. This stimulated lipolysis significantly more in both KD1 and KD2 cell pools in comparison with the control cell pool. Enhancement of lipolysis was observed both in the presence and absence of isoproterenol (Fig. 3A, *III*). Interestingly, we also observed that Angptl8 mRNA expression in naive 3T3-L1 adipocytes was dampened by both isoproterenol and forskolin treatments, known stimulators of adenylate cyclase increasing protein

kinase A activity and lipolysis (Fig. 3B, *III*). Hence, we analysed the genes responsible for the regulation of lipolysis. mRNA expression levels were measured for Angptl4, Pnpla2, Pnpla3 and hormone sensitive lipase (HSL) after inducing lipolysis for 2 hours. Angptl4 mRNA expression was significantly elevated in both KD1 and KD2 cell pools relative to non-targeting controls, while HSL and Pnpla2 expression were not altered. We observed an elevated expression of Pnpla3 in KD1, but however not in KD2 (Fig. 4A, *III*). Analysis of relevant genes involved in fatty acid oxidation, Ucp1, Cpt1a, Cpt1b and PGC-1 $\alpha$ , revealed a significant increase in KD1 and a similar trend in KD2. Cpt1a and Cpt1b were upregulated in both KD1 and KD2 cell pools (Fig. 4B, *III*). We also observed the increased expression of Leptin mRNA expression in both Angptl8 KD1 and KD2 cells (Fig. 4B, *III*).

## 6. Discussion

In this study, we focused on identifying novel regulators of adipose tissue lipid homeostasis with special emphasis on miRNA-based regulation. With three independent projects, we have discovered the miRNA species miR-192\* and miR221-3p as modulators of lipid metabolism and identified ANGPTL8 as a regulator of adipocyte lipolysis.

We first started investigating miR192\*(miR192-3p) based on an interesting correlation: Expression of miR-192\* in VAT of morbidly obese subjects correlated negatively with serum triglyceride content and positively with HDL-cholesterol and AST. Expression of miR-192\* correlated negatively with BMI of another female patient cohort with lower BMI. miR-192\* originates from the 3' arm of miR-192, a well-studied miRNA and shown to be associated in liver disease<sup>247,248</sup>, type 2 diabetes<sup>15,26</sup> and cancers<sup>249,250</sup>. However, studies on miR-192\* expression and function are scarce. We showed that stable overexpression of miR-192\* in cultured SGBS adipocytes reduced TG accumulation and affected adipogenic differentiation as established by the low TG content and downregulation of adipogenic markers proteins aP2<sup>251</sup> (FABP4) and perilipin 1<sup>252,253</sup>. Neither aP2 nor perilipin are direct targets of miR-192\* but their reduced expression or ablation exerts major physiological changes, for example, T87C polymorphism in humans at aP2 locus causes reduced gene expression which lowers serum TG levels resulting in considerably reduced risk for coronary heart disease and type 2 diabetes relative to subjects homozygous for the wild-type allele<sup>254</sup>. Perilipin 1 is a lipogenic factor. Deletion of perilipin 1 in mice resulted in reduced adipose tissue mass<sup>255,256</sup>. It is essential for normal deposition and retention of TG in lipid droplets. Through transcriptome profiling and pathway analysis of SGBS adipocytes expressing miR-192\* or NT-miR, two key lipid regulatory pathways 'LXR/RXR activation' and 'LPS/IL-1 mediated inhibition of RXR function', were found to be altered. LXR/RXR initiation is essential for adipogenic differentiation and TG storage is

mediated by PPAR $\gamma$  or C/EBP $\alpha$  in both 3T3-L1 and SGBS cell systems<sup>257-261</sup>. Many lipogenic genes from 'LPS/IL-1 mediated inhibition of RXR function' pathway like SREBF1, FABP7, ACSL3 and ACSL6 were found to be downregulated in SGBS cells expressing miR-192\*. We confirmed the downregulation of SCD1, SCARB1 (SR-B1) and ALDH3A2 at protein level by western blotting. More importantly, we showed that SCD and ALDH3A2 are direct targets of miR-192\*. SCD1 is a highly regulated enzyme that catalyses the synthesis of monounsaturated fatty acids (MUFAs) from saturated fatty acids derived either from diet or *de novo* lipogenesis. MUFAs are major lipid components of TG, cholesterol esters and phospholipids<sup>262</sup>. Oleate is the primary product of SCD1 and comprises up to 45% of free fatty acids in human adipose tissues<sup>263</sup>. SCD1 is abundantly expressed in liver and adipose tissue<sup>264</sup> and reported in other lipogenic tissues<sup>265</sup>. SCD1 has been shown to regulate lipid synthesis and oxidation, hormonal signalling, thermogenesis and inflammation. SCD1 knockout mice have shown lowered adiposity, increased insulin sensitivity and are not affected by diet induced obesity<sup>266</sup>. Moreover, lipogenic diet was unable to induce TG synthesis in their liver<sup>267</sup>. Liver specific SCD1 knockout mice were resistant to hepatic steatosis and diet-induced obesity. Adipose specific SCD1 knockout mice show enhanced GLUT1-mediated glucose uptake and lowered expression of adiponectin in AT<sup>268</sup>. Blocking SCD1 expression in 3T3-L1 adipocytes reduced expression of several lipogenic genes resulting in reduced TG synthesis<sup>269</sup>. SCD1 has been shown to regulate inflammatory response via modulating DNA methylation in 3T3-L1 adipocytes<sup>270</sup>. SCD1 expression was relatively reduced in morbidly obese human subjects compared to overweight subjects<sup>271</sup>. The above data together suggest an important role of SCD1 in lipid synthesis and metabolism. Inhibition of SCD1 expression by miR-192\* in the VAT could effectively hamper TG synthesis resulting in an overflow of non-esterified fatty acids and inflammatory stimuli towards the liver.

Aldehyde dehydrogenase (ALDH3A2 or FALDH), is a fatty acid dehydrogenase which facilitates the detoxification of aldehydes from lipid peroxidation events by converting long chain aliphatic aldehydes to fatty acids<sup>272</sup>. ALDH3A2 is elevated by insulin, and its expression is

downregulated in an animal model with insulin resistance<sup>273</sup>. Its suppression may lead to pathologic effects of altered fatty aldehyde metabolism like lipotoxicity, which may lead to insulin resistance and proinflammatory signaling in VAT. Scavenger receptor class B, type I (SR-B1, gene SCARB1) serves as a receptor for high-density lipoproteins (HDL)<sup>274</sup>. It facilitates the selective uptake of lipids from HDL and is expressed in several types of tissues. Its role in adipocytes is not well explored. However, it has been shown to facilitate the anti-inflammatory action of HDL and to induce glucose uptake by HDL in adipocytes<sup>275,276</sup>. Inhibition of SCARB1 expression may thus influence the anti-inflammatory and pro-lipogenic functions of HDL in VAT.

High TG and low HDL-cholesterol are hallmarks of CVD, obesity and metabolic syndrome<sup>277,278</sup>. We found a negative correlation between the expression of miR-192\* and serum TG, and a positive correlation between miR-192\* and HDL cholesterol in VAT of morbidly obese subjects. However, there was no correlation found with LDL-cholesterol. This tempts us to speculate on a possible direct or indirect function of miR-192\* in regulating HDL and/or VLDL production by the liver which is physiologically connected with VAT via portal circulation<sup>279</sup>. Alternatively, it could affect VLDL metabolism to successively influence HDL-cholesterol<sup>278,280</sup>. As a yet another interesting result, we found both miR-192\* and miR-192 species in the AT of morbidly obese subjects positively correlated with AST, a liver enzyme routinely used as a biomarker for hepatic damage. This supports the hypothesis that the increased expression of miR-192\* could result in an abnormal VAT fat storage and elevated flux of lipid species towards liver, which may result in liver dysfunction<sup>281,282</sup>.

In a female cohort (cohort 2) with a lower BMI we did not observe any correlation between miR-192\* with TG, HDL and AST, strongly suggesting that the correlations are tightly associated with metabolic status of the morbidly obese cohort. Like in the morbidly obese cohort, we did not notice correlation with LDL cholesterol. However, we observed negative correlation between VAT miR-192\* and BMI of (less obese) cohort 2 which is consistent with the cultured adipocyte results

suggesting that miR-192\* downregulates adipogenesis. No similar tendency or correlation was observed in the morbidly obese cohort (cohort 1). We envision that morbid obesity represents an extreme metabolic condition where subtle regulatory changes could not be detectable at this point. Comparison of miR-192\* levels between the two cohorts did not uncover any alterations. However, when cohort 2 (lower BMI group) was subdivided based on BMI <30 or >30, lower miR-192\* expression was seen in the BMI >30 subgroup, further supporting the concept that miR-192\* reduces lipid storage in adipocytes.

In the fourth study, we show that ANGPTL8 expression in human SAT and VAT shows a strong positive correlation with hallmark insulin-regulated or adipogenic markers. ANGPTL8 is one of the insulin sensitive genes involved in lipid metabolism reported in our previous studies<sup>33,34</sup>. This motivated us to investigate its regulation in adipose tissue. We found that ANGPTL8 expression positively correlated with major insulin sensitizers GLUT4 and ADIPOQ suggesting that ANGPTL8 expression in adipocytes might also contribute to insulin sensitivity, and it is consistent with the notion that ANGPTL8 promotes white adipocyte lipid storage<sup>27,213,283</sup>. We also found that ANGPTL8 expression positively correlated with important lipogenic genes like DGAT1 and FASN.

There are several controversial reports on serum ANGPTL8 level in insulin resistant condition and subjects with T2D. Most reports suggest that ANGPTL8 levels are elevated under these conditions<sup>31,32,198,284,285</sup>. On the contrary, some studies including that of Gomez-Ambrosi *et al.* indicate that ANGPTL8 expression is rather decreased in patients with T2D and dyslipidemia<sup>198,286</sup>. Also, data from Pascual-Corrales *et al.*<sup>287</sup> suggests that serum ANGPTL8 is elevated after bariatric surgery and not due to diet-induced weight loss. This observation is consistent with our findings on plasma ANGPTL8 levels in a bariatric surgery cohort, where we found that ANGPTL8 expression is downregulated upon adipocyte culture under inflammation-mimicking conditions and correlates negatively with an inflammatory marker mRNA in morbidly obese subjects. Till now detection of ANGPTL8 protein levels in plasma has not been well established and the regulation of its secretion, tissue source



and protein turnover are main areas to be studied considering the suggestion that the liver could be the main source of circulating ANGPTL8<sup>29,288</sup>. The expression of ANGPTL8 was heavily repressed during inflammatory conditions, as well as the expression of GLUT4, ADIPOQ, DGAT1 and FASN. Also, ANGPTL8 gene expression displayed a significant negative correlation with the AT inflammatory marker PLA2G7<sup>33,244-246</sup> in morbidly obese pre-bariatric surgery patients. It is established that inflammation is a key contributor to adipocyte insulin resistance<sup>96,289,290</sup>. Therefore, we speculate that suppression of ANGPTL8 expression under inflammatory conditions may significantly affect adipocyte lipid metabolism and could also be driving adipocytes towards insulin resistance upon AT inflammation. miRNAs are actively involved in regulating various biological events including metabolism. Upregulation of many miRNAs have been reported during inflammation in obese and/or diabetic subjects<sup>160,291,292</sup>. We investigated whether inflammation could regulate ANGPTL8 expression via miRNAs. Hence, we screened the 3'UTR sequence of ANGPTL8 and discovered many potential miRNA binding sites, out of which we shortlisted three miRNAs: miR-107, miR-143-3p and miR-221-3p based on their previously reported involvement in insulin resistance of AT<sup>192,293,294</sup>. Among these three, miR-221-3p was significantly increased under inflammatory stimulus in primary adipocytes and positively correlated with the prominent inflammatory marker PLA2G7 in the pre-bariatric surgery cohort. Hence, we focused our study on miR-221-3p. Using dual luciferase assay with Luc-ANGPTL8 3'UTR constructs and miR-221-3p mimics, we demonstrated that miR-221-3p directly binds the ANGPTL8 3'UTR. This result was further strengthened by the follow-up experiment where miR-221-3p significantly suppressed the endogenous ANGPTL8 protein expression and TG storage in SGBS adipocytes. Several studies have shown that miR-221, a 5' product of miR-221 pre-miRNA also suppresses adiponectin receptor-1 causing altered adiponectin signalling and insulin sensitivity of adipocytes<sup>192</sup>. miR-222, additional member of the miR-221/222 family, suppresses GLUT4 expression and may eventually affect insulin sensitivity in gestational diabetes<sup>193</sup>. This suggests that the miR-221/222 family plays a pivotal role in the regulation of adipocyte insulin sensitivity and

metabolism<sup>158,191</sup>. We found a negative correlation between miR-221-3p and ANGPTL8 expression in SAT of morbidly obese subset of cohort III prior to bariatric surgery, which, interestingly, disappeared after surgery-induced weight loss, concurrent with improved dyslipidaemia and insulin sensitivity along with lowered miR-221-3p expression. These substantial evidences suggest that miR-221-3p is a significant regulator of ANGPTL8 under physiological conditions like severe obesity and inflammation. Improved inflammatory and metabolic condition along with loss of the above correlation after weight loss suggests that, under these conditions, additional mechanisms other than miR-221-3p might dominate in the regulation of ANGPTL8 expression. In the future, the correlation of AT miR-221-3p and ANGPTL8 should be studied at the protein level.

In this study, we investigated the function of ANGPTL8 in adipocytes. First, we generated a pool of stable *Angptl8* knock-down (*Angptl8* KD) 3T3-L1 adipocytes through lentivirus-mediated shRNA expression. *Angptl8* KD resulted in reduced TG storage. Several studies have previously implicated the upregulation of ANGPTL8 expression during adipocyte differentiation<sup>27,295</sup> and upon treatment with insulin which facilitates the storage of TG<sup>27,34,295</sup>. In a formerly published study by Ren et al<sup>27</sup>, transient siRNA-mediated knockdown of *Angptl8* in 3T3-L1 adipocytes led to a defect in adipocytic differentiation. Our stable *Angptl8* knockdown 3T3-L1 adipocytes displayed rather a mild reduction in TG storage. However, this effect could be the result of a delicate defect in terminal differentiation of the adipocytes.

Lipidome analysis of *Angptl8* KD cells revealed a marked decline in alkyl-PCs and PE plasmalogens with an ether-linked FA chain, generally in the *sn-1* position. Biosynthesis of ether phospholipids is initiated by a peroxisomal enzyme cascade<sup>296</sup>. Hence, we speculated that loss of *Angptl8* may result in a malfunction of peroxisomes. Alkyl glycerol lipids serve as natural adipocyte differentiation factors and external addition of these lipid species promotes adipocytic differentiation in 3T3-L1<sup>297</sup>. Similarly, *Pex7* KO mice lacking plasmalogens show lower body fat with characteristic small LDs in both brown and white fat<sup>298</sup>. Further, we observed reduced amounts of saturated PCs and PEs (rare lipid species).

There were no significant changes found in the total saturation degree of phospholipids. The reason for this observation remain elusive. However, a lowered content of ether phospholipids tends to reduce cellular membrane fluidity<sup>299,300</sup>, and reduction of saturated PCs and PEs could be a compensatory mechanism to counteract a change in membrane fluidity. One of the important findings of Angptl8 knockdown was enhanced lipolysis in 3T3-L1 adipocytes, suggesting an endogenous function of Angptl8 as an inhibitor of lipolysis. This in line with the earlier reports where Angptl8 is highly induced by insulin<sup>34,301</sup>. Suppression of stored TG hydrolysis may occur instantaneously even during the process of FA deposition as TGs in WAT. This suggests that Angptl8, analogous to its function in the circulation<sup>29,213</sup>, could act as an intra- cellular inhibitor of lipolysis. We did not observe any changes in mRNA expressions of the lipolytic genes, ATGL and HSL. However, a mild increase of Angptl4 mRNA expression was detected. Angptl4 stimulates adipocyte lipolysis<sup>302</sup>; hence its upregulation could have been resulted in increased lipolysis. Among the two Angptl8 knockdown (KD1 and KD2) cell pools, KD1 showed relatively stronger lipolysis than KD2, and also a marked elevation of PNPLA3 message was noted. PNPLA3 has been shown to increase lipolysis and TG remodelling in hepatocytes<sup>303,304</sup>. Elevated expression of PNPLA3 could have caused increased in lipolysis in KD1. At the moment, we are unable to conclude the reason behind the observed discrepancy between KD1 and KD2. However, we speculate that putative off-target effects of the shRNA could have alleviated the lipolysis effect in KD2. Plasmalogens serve as a source of prostaglandin E2 (PGE2) which serves as an inhibitor of lipolysis in adipocytes<sup>305,306</sup>. Reduced amounts of PGE2 increase the cellular cAMP leading to enhanced lipolysis<sup>307</sup>. Also, patients with Zellweger syndrome, a genetic peroxisome disorder, display a plasmalogen deficiency<sup>308</sup>.

We noticed a strong induction of the leptin gene in the Angptl8 KD cell pools under basal conditions. However, the mRNA was barely expressed under the lipolytic conditions employed. This outcome is debatable since PGE2 has been reported to increase leptin secretion by adipocytes<sup>306,309</sup>. Hence, the observed induction of leptin mRNA could signify a compensatory response to downregulate the synthesis of PGE2.

Alternatively, knockdown of Angptl8 might influence leptin expression via another, as yet unknown mechanism. Along with the lipolysis phenotype, we also detected several genes associated with FA oxidation significantly induced in the Angptl8 KD cell pools. This is in line with the observed upregulation of lipolysis releasing free FAs, which can be utilized as an energy source through oxidation and further must be degraded to minimize the FFA-mediated cellular lipid toxicity<sup>310</sup>.

## 7. CONCLUSIONS

Evidence suggests that miRNAs are crucial for adipose tissue function. Defects in miRNA expression cause impairment of fundamental functions of adipocytes, such as differentiation, metabolism, and signalling. This influences the AT cellularity and size, which in turn leads to unusual fat accumulation and results in the metabolic disorders. miRNAs regulate AT endocrine function by regulating the expression of adipokines and their signalling pathways. Moreover, miRNAs are actively involved in the crosstalk between adipocytes and immune cells within AT.

In this thesis, we have studied the expression and characterized the function of miR-192\* in the VAT of human cohorts and in cultured adipocytes, respectively. VAT miR-192\* expression negatively correlated with serum TG and positively with HDL in morbidly obese group and negative correlation with BMI was recorded in a less obese cohort. Stable overexpression of miR-192\* in cultured adipocytes resulted in reduced TG accumulation and reduced the expression of key adipogenic and lipogenic genes. miR-221-3p, which had previously been shown to be associated with metabolic disorders, was shown to be induced upon inflammatory conditions in adipocytes and to directly target ANGPTL8. We showed that ANGPTL8, a well-documented regulator of lipid metabolism, negatively correlated with miR-221-3p and the AT inflammatory gene PLA2G7 in SAT of a morbidly obese human cohort. Depletion of ANGPTL8 in cultured adipocytes resulted in the enhancement of intracellular lipolysis and an altered ether phospholipid content. With the gathered evidence, we have shed light on the roles of miR-192\*, miR-221-3p and ANGPTL8 as regulators of adipose tissue lipid metabolism.

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